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(57) Abstract

This invention relates to a method of maturation of dendritic cells *in vitro* a serum–free medium, and to methods of treatment using the mature dendritic cells.

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CELLULAR ADJUVANT

This invention relates to a cellular adjuvant and to a method of production thereof. In particular, the invention relates to activation of dendritic cells to mature, functional dendritic cells for use as a cellular adjuvant in treatment of neoplastic disease.

BACKGROUND OF THE INVENTION

The recent identification of tumour-related cytotoxic T lymphocyte (CTL) epitopes together with reports of clinical anti-tumour responses by adoptive transfer of autologous peptide-specific CTL has raised the possibility that peptide-based vaccination strategies may be useful for cancer treatment (Boon *et al*, 1994; Kawakami *et al*, 1995). The outstanding ability of dendritic cells (DC) to function as antigen-presenting cells (APC) makes them attractive as potential cellular adjuvants for activating and expanding peptide-specific anti-tumour $CD8^+$ CTL (Bakker *et al*, 1995; Bhardwaj *et al*, 1994; Macatonia *et al*, 1991).

However, the lack of lineage-specific surface markers and the multitude of ways of purifying or growing DC have made it difficult to characterize these cells definitively. DC are usually identified by their typical morphology, characterized by the presence of dendrites or membrane processes, and their characteristic surface antigen expression pattern (positive for CD1a, CD11c, CD40, CD80, CD83, CD86, HLA-A,B,C and HLA-DR, and negative for mature leukocyte markers of other lineages) (Macatonia *et al*, 1991; Szaboles *et al*, 1995; Caux *et al*, 1992).

To obtain DC for clinical application, a number of sources and culture methods has been proposed. DC can be purified from peripheral blood (Steinman and Young, 1991). They can also be grown in culture from peripheral blood mononuclear cells (PBMC) in the presence of cytokines such as granulocute/macrophage-colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) (Romani *et al*, 1994;

Inaba *et al*, 1992). Another technique for growing DC involves harvesting $CD34^+$ cells from umbilical cord blood, bone marrow or cytokine-mobilized peripheral blood progenitor cells and culturing these cells in GM-CSF and tumour necrosis factor-alpha (TNF-α) with or without IL-4 (Szabolcs *et al*, 1995; Caux *et al*, 1992; Romani *et al*, 1994; Santiago *et al*, 1992; Mackensen *et al*, 1995; Siena *et al*, 1995; Herbst *et al* , 1996; Strunk *et al*, 1996). To increase yields, the addition of stem cell factor (SCF) either alone or together with a cocktail of cytokines (IL-1b, IL-3, IL-6, and erythropoietin) has been tried (Szabolcs *et al*, 1995; Mackensen *et al*, 1995; Santiago Schwarz *et al*, 1995).

Hitherto, none of these methods has proved successful in providing a purified population of mature, functional DC in sufficient numbers for clinical use, although clinical trials using DC from PBMC have commenced. Methods of expanding the DC population in culture have therefor been sought.

Methods of growing DC usually utilize either foetal calf serum (FCS) (Szabolcs *et al*, 1995; Caux *et al*, 1992; Mackensen *et al*, 1995; Siena *et al*, 1995) or pooled human serum (Santiago Schwarz *et al*, 1992; Siena *et al*, 1995; Santiago Schwarz *et al*, 1995) in the culture medium. However, the use of serum is undesirable for several reasons. Given that both peripheral blood DC precursors and Langerhans cells (LC) take up and process soluble antigens (Sallusto *et al*, 1995; Stossel *et al*, 1990), DC that differentiate from cultured $CD34^+$ cells would be expected to take up and process proteins derived from serum present in the medium. Indeed, when DC cultured in the presence of FCS were used to stimulate CTL *in vivo*, an overwhelming generation of cytolytic activity against bovine antigens was observed (Porgador and Gilboa, 1995). Donor serum may also constitute an infectious risk for patients if DC are to be used clinically, and the use of xenogeneic serum is usually considered to be undesirable.

Finally, serum contains growth factors, and thus its presence might interfere with the study of the role of cytokines in DC differentiation and maturation, even though this may not be of great relevance to ultimate production of a cellular adjuvant for clinical use.

We have now developed a method to generate DC which function as potent APC for both allogeneic T lymphocytes and autologous, peptide-specific CTL, using $CD34^+$ progenitors under serum-free conditions. To our knowledge we are the first to show that DC can be generated from $CD34^+$ progenetors in the absence of the stem cell-expanding cytokines stem cell factor, IL-3, flt-3L or other expensive additives such as TGF-β.

SUMMARY OF THE INVENTION

In one aspect, the invention provides a method of inducing the maturation of dendritic cells *in vitro*, comprising the step of culturing mononuclear cells in a serum-free medium in the presence of a Type I interferon following an initial phase of growth in the presence of GM-CSF, TNF-α and IL-4. Preferably the mononuclear cells are $CD34^+$ haematopoietic progenitor cells.

The cells which are cultured may be peripheral blood progenitor cells, for example obtained by leukapheresis, or bone marrow progenitor cells. More preferably the starting cell population is also enriched for $CD34^+$ cells prior to culture.

Preferably the cells are cultured for 14 to 35 days, more preferably from 14 to 28 days, and even more preferably for 14 to 17 days.

Preferably GM-CSF, TNF-α are present in the culture medium for the whole period of culture, and preferably the Type I interferon is present commencing at day 13 of culture. Preferably IL-4 is present commencing at day 6, or is present for the whole period of culture.

Any Type I interferon may be used, for example interferon-α, interferon-β, interferon-ω, or consensus

interferon (Infergen; Amgen, Inc., Thousand Oaks, California). Preferably the interferon is IFN-α2a, IFN-α8 or IFN-β.

If peripheral blood mononuclear cells are to be used, it may be advantageous to pre-treat the patient or animal from which the cells are to be obtained with a cytokine such as G-CSF or GM-CSF prior to leukapheresis in order to mobilise bone marrows stem cells into the circulation. Enrichment of between 10- and 1000-fold of stem cells in the circulation is desirable. In the absence of such priming, there may be insufficient stem cells in the circulation, and bone marrow cells are then preferably used.

Optionally the cells may also be cultured in the presence of stem cell factor (SCF). Where SCF is used, it is preferably present throughout the culture period.

Maturation of the dendritic cells may be monitored using morphological markers such as the presence of dendritic processes at the cell surface, and the presence of immunological markers such as CD40, CD80, CD83, CD86, and CMRF44.

The matured dendritic cells produced by the method of the invention are non-phagocytic, and have potent antigen-presenting activity. They are capable not only of stimulating multiplication of allogeneic lymphocytes in the mixed leukocyte reaction (MLR), but also of stimulating autologous, peptide-specific cytotoxic T lymphocytes in the mixed leukocyte peptide culture assay system (MLPC).

Therefore in a second aspect the invention provides a method of enhancing the antigen-presenting capacity of dendritic cells, comprising the step of exposing dendritic cells to a Type I interferon. Any Type I interferon can be used, as described above. Interferon can be administered *in vivo*, in order to activate either the recipient's own resident dendritic cells, including Langerhans cells or other tissue antigen-presenting cells, or antigen-presenting cells which have been infused or

injected and are subsequently activated *in vivo* by systemic administration of interferon. Where interferon is administered *in vivo* it may be delivered at the site of dendritic cell administration, as set out below or may be administered in such a way as to spread systemically through the body. The interferon it may be given in conjunction with other cytokines which induce proliferation of DC; for example interferon may be given in conjunction with, ie sequentially or simultaneously with, GM-CSF or GM-CSF and IL-4. Preferably dendritic cells are exposed to interferon *in vitro*, as described above.

In a third aspect, the invention provides a method of preparation of a cellular adjuvant for treatment of a neoplastic disease or a disease caused by an infectious agent, comprising the steps of maturing dendritic cells *in vitro* as described above, and exposing the matured dendritic cells to an antigen derived from the neoplastic cells (*ie.* a tumour-associated antigen) or from the infectious agent, respectively.

The infectious agent may be a bacterium, a virus, a yeast or a parasite.

The antigen may be a protein, peptide, polysaccharide, or nucleic acid. The dendritic cells may be transfected with the antigen, or may be incubated *in vitro* with the antigen.

The cellular adjuvant of the invention may be used in the treatment of a neoplastic disease, in order to provoke or stimulate an anti-tumour response. Thus the invention provides a method of treatment of neoplastic disease, comprising the step of administering an effective dose of the cellular adjuvant according to the invention to a patient in need of such treatment. As an alternative to transfection of dendritic cells with antigen or incubation of dendritic cells with antigen in culture, matured dendritic cells according to the invention may be administered to the patient together with the antigen. Any suitable route of administration may be used, for example

subcutaneous, intravenous, intratumour, or intra-lymphatic. Preferably the dendritic cells are autologous, *ie.* from the patient. The neoplastic cell antigen may also be derived from the patient's own tumour cells.

Similarly the cellular adjuvant of the invention may be used in the treatment of disease caused by an infectious disease, using an antigen derived from the infectious agent as discussed above.

The method of the invention may also be used to generate clones of activated tumour-associated T cells. Thus, in another aspect the invention provides a method of producing a population of activated tumour-associated T cells, comprising the steps of harvesting dendritic cells and lymphocytes from peripheral blood or bone marrow of a patient suffering from a tumour, maturing dendritic cells according to the method described above, and culturing the dendritic cells and the lymphocytes, either separately or together, in the presence of a tumour-associated antigen, and optionally in the presence of a cytokine. Preferably the cytokine is a type I interferon, more preferably interferon-α.

This aspect of the invention also provides a method of treatment of a patient suffering from a tumour, comprising the step of administering an effective dose of dendritic cell-activated, tumour-associated T cells to a patient in need of such treatment. Alternatively, this aspect of the invention provides a method of treatment of a pathological condition, comprising the step of administering an effective dose of mature dendritic cells having the functional and antigenic characteristics as defined herein to a mammal in need of such treatment, together with or subsequently to adminstration of an antigen associated with the condition. The pathological condition may be a neoplastic disease, in which case the antigen is derived from neoplastic cells alternatively the condition may be an infectious disease, and the antigen is derived from the infectious agent.

In these last two aspects of the invention it is particularly contemplated that the tumour antigen is a cancer-associated peptide such as Melan A, tyrosinase, GP100, or an antigen of the MAGE family, or a member of the family of cancer antigens, such as ESO-I or a cancer testis antigen detectable by the SEREX method. The antigen may alternatively be derived from cancer cells or may be autologous, irradiated tumour cells, optionally in conjunction with GM-CSF.

Another important aspect of the invention provides a method of using a Type I interferon as an adjuvant for peptide-based anti-tumour vaccination strategies. In one embodiment of this aspect, a Type I interferon is administered locally or systemically at the same time as, or 1 to 3 days after vaccination of a patient in need of such treatment with a tumour peptide or tumour cell lysate, optionally in conjunction with GM-CSF. In a second embodiment, this aspect of the invention provides a method using a type I interferon as an adjuvant for vaccination against an infectious agent. Preferably the infectious agent is a bacterium, a virus, a yeast or a parasite.

It will be clearly understood that while the invention is defined above with reference to treatment of human patients, it is also applicable to non-human mammals and therefore to veterinary treatment, including but not limited to treatment of domestic, companion or zoo animals.

Brief Description of the Figures

Figure 1 shows a comparison of antigen expression and morphology of cultured cells after 14 and 28 days of culture,

A) Comparison of CD1a, CD83 and CD86 expression within the large-sized (high forward scatter) population at day 14 (—) and day 28 (▬). DC cultures were grown in GM-CSF (10 ng/mL), TNF-α (20 ng/mL) and IL-4 (500 U/mL), and flow cytometry was performed at the

indicated times to assess antigen expression. One representative experiment is shown. x-axis, fluorescence intensity (logarithmic scale); y-axis, cell number.

B) Cytospin preparations of unseparated, cultured cells at day 14 (I) and day 27 (II). Staining was performed by May Grunwald-Giemsa stain. Original magnification for both panels: x200.

Figure 2 shows the immunophenotype of DC after 28 days in serum-free culture.

Analysis of DC by flow cytometry at day 28. Culture conditions as in Figure 1A. DC (23% of total cells) were gated on the basis of high forward and side scatter properties as shown in A). In B), staining of this cell population with the indicated antibodies (filled histograms) is shown. The unfilled histograms represent staining with the control antibody.

Figure 3 shows the assessment of antigen-presenting function of mature DC.

A) MLR. Stimulation index of DC cultures and autologous PBL in the allogeneic MLR. The points represent mean±SEM. DC cultures were grown as described in Figure 1A for 26-28 days before inclusion in the assay (n=27). PBMC controls were obtained by thawing $CD34^-$ cells, originally isolated and frozen at the time of $CD34^+$ cell separation (300-10,000 stimulators, 100,000 responders) (n=3). Thymidine incorporation after 5 days was measured and divided by the Thymidine uptake of responder cells alone to calculate the stimulation index.

B) MLPC. Comparison of the ability of sorted DC and autologous PBMC to generate autologous, peptide-specific CTL (n=3). DC cultures were grown as described in Figure 1A. At day 27 of culture, DC were sorted by FACS, and DC and PBMC were pulsed with influenza matrix peptide and co-cultured with 10^6 autologous PBMC in an MLPC. Numbers of stimulators are shown in the figure. At day 21 of the MLPC, cells were tested for their ability to lyse matrix peptide-labeled T2 cells in a ^{51}Cr release assay.

Background lysis against T2 cells without peptide was always < 20% of maximal release, and was subtracted from experimental lysis percentages to give specific lysis results as shown here.

Figure 4 shows the cytokine optimization within serum-free DC cultures.

A) Effect of GM-CSF concentration and IL-4 on the percentage of CD1a+ cells in culture at day 14. Cultures containing GM-CSF (50 ng/mL) alone (n=2); TNF-α (20 ng/mL) and IL-4 (500 U/mL) without GM-CSF (n=1); GM-CSF (10, 50 and 200 ng/mL) together with TNF-α (20 ng/mL) and IL-4 (500 U/mL) are compared. Each data set represents a single subject. CD1a expression within cultures was assessed by flow cytometry at day 14.

B) Effect of TNF-α concentration on the percentage of CD86+ cells in culture. Cultures containing TNF-α (2.5, 20, 100 ng/mL), GM-CSF (10 ng/mL) and IL-4 (500 U/mL) are compared (n=3). CD86 expression was assessed by flow cytometry at day 28.

C) Effect of IL-4 on the proportion of CD86+ cells between days 21 and 28 of culture. Cultures containing GM-CSF (50 ng/mL), TNF-α (20 ng/mL) with or without IL-4 (500 U/mL) are compared. The results of eight individual experiments are shown (mean±SEM: with IL-4: 39±7%, without IL-4: 25±6%). CD86 expression was assessed by flow cytometry between days 21 and 28.

Figure 5 shows the effect of human serum on development of CD14-, CD83+ DC at day 28. Results of one representative experiment are shown (CD14: n=4; CD83: n=2).

A) Cultures contained GM-CSF (50 ng/mL) and TNF-α (20 ng/mL).

B) as in A) with 10% human serum from day 0; x-axis: fluorescence intensity (logarithmic scale); y-axis: FSC.

Figure 6 shows the cell yield kinetics and early differentiation in serum-free cultures.

A) Time course of overall cell yields in serum-free conditions is shown. Results are represented relative to the number of $CD34^+$ cells present at the initiation of the cultures (10^5 cells in 100µl: ●, ■; $2x10^5$ cells in 100 µl: ▲,▼,◆; all derived from leukapheresis products). Cells were maintained in the following cytokines: GM-CSF (40ng/ml), IL-4 (500U/ml) and TNF-α (20ng/mL).

B) Cell differentiation within the first three days. Up-regulation in CD33 expression by the $CD34^+$ cells is observed between the start of culture (day 0) and day 3. Culture conditions as in A. Flow cytometry was performed at the indicated times to assess antigen status. One representative experiment is shown (n=4).

Figure 7 shows the time course of antigen expression over days 3-17).

10^5 $CD34^+$ cells were cultured in 100 µl cultures in 8 replicates in 96-well plates, and one well was harvested every second day for FACS analysis. Culture conditions were as in Figure 1A. Vertical rows of dot plots represent antigen expression in one well at one time point. Total cell population of one representative experiment is shown (n=3, all derived from leukapheresis products). Vertical gates to distinguish positive populations were set according to control Ab (first horizontal lane). Horizontal gates were set to distinguish large and small cell populations.

Figure 8 shows the morphology of sorted $CD1a^+$ DC and $CD1a^-$ cells at day 14

A) Early DC stages showed temporary plastic adherence. Phenotype of $CD11b^{bright}$ DC at day 14, 24 hours after sorting. Original magnification (OM) x180.

B) I: Electron micrograph picture of sorted $CD1a^+$ dendritic cells (day 14). The bar indicates 1.5 µm,

II: Birbeck Granules in sorted $CD1a^+$ DC (day 14). The bar indicates 0.23 µm,

III: Mature DC (day 28) did not contain Birbeck Granules. The bar indicates 2.1 μm.

C) Electron micrograph picture of sorted CD1a$^-$ cells.

I: mononuclear phagocytic cell, containing phagocytic vacuoles. The bar indicates 1.2 μm.

II: granulocyte with segmented nucleus. The bar indicates 1.2 μm.

III: mononuclear, non-phagocytic cell. The bar indicates 1.8 μm.

Figure 9A shows the DC surface phenotype on days 14 and 28 by flow cytometry.

Phenotypic changes during DC maturation: CD34$^+$ cells were cultured in GM-CSF, TNF-a and IL-4 and analyzed by flow cytometry after 14 days and 28 days. Cultures were gated to examine only the large cells (20% of total cells). Comparison between day 14 (——) and day 28 (———) is shown in a representative experiment. The position of the control cells labeled with non-specific antibody (IAG-11) is indicated by the horizontal line. x-axis: fluorescence height (log scale), y-axis: relative cell number.

Figure 9B shows CD11b expression in our representative culture at three different time points. Cultures and flow cytometry were performed as described for Figure 9A.

Figure 10 shows the differentiation of subpopulations sorted at day 13.

A) Expression of CD11b on total cells after 13 days in serum-free culture. (Culture conditions as in Fig.1A). Three subpopulations were sorted according to FSC and CD11b expression: I=small, CD11b$^-$ cells; II=small, CD11b$^+$ cells; III=medium/large, CD11b^{bright} cells.

B) Differentiation of subpopulation I (small, CD11b$^-$) cultured until day 18 in GM-CSF, TNF-α and IL-4.

C) Differentiation of subpopulation II (small, CD11b$^+$) cultured until day 18 in GM-CSF, TNF-α and IL-4.

D) Differentiation of subpopulation III (medium/large, $CD11b^{bright}$) cultured until day 18 in GM-CSF, TNF-α and IL-4.

Figure 11 shows the allostimulatory capacity of DC cultured in GM-CSF, TNF-α and IL-4 for days 14 and 28 using mixed leukocyte reaction

The allostimulatory capacity of 14 individual unsorted DC cultures was directly compared after 14-15 days (■) and 28 days (▼) using the same allogeneic responder cells for both time points. DC were cultured in GM-CSF, TNF-α and IL-4, irradiated and co-cultured with 10^5 PBMC in triplicate wells. ^{3}H-thymidine uptake was measured after 5 days. Thymidine incorporation of responder cells alone was 1600±350 cpm. Results are shown as the mean±SE of 14 individual experiments (* $p<0.05$, ** $p<0.01$).

Figure 12 shows the superior effectiveness of mature Dendritic Cells expressing low levels of CD11b in stimulating CD8+ peptide-specific lymphocytes, compared to immature DC expressing high amounts of CD11b.

Figure 13 shows the effect of addition of human serum to cultures containing GM-CSF, TNF-α and IL-4 on the expression of CD1a and CD86. Cultures were split 3 days before analysis and human serum was added into one half culture.

A) Normal development of serum-free cultures: accumulation of a population of large sized, CD1a+ DC which did not express CD86.

B) Addition of human serum 3 days previous to analysis by flow cytometry. Similar expression of CD1a and CD86 in culture suggesting the quick maturation of CD1a+ DC in response to activating factors in human serum.

Figure 14 shows the screening for cytokines for activating effects on DC.

$CD34^+$ cells were cultured in 96 well plates in GM-CSF, TNF-α and IL-4 (GTI). At day 14, cytokines were added at the concentrations shown in Table 3. Cultures were analyzed by flow cytometry at day 17. The proportion

of activated DC (HLA-DR^{+++} and/or CD86^{+}) was calculated referring to the mean expression in multiple control cultures (GTI) as 100%. SDs were calculated for all protocols and a no-difference interval of two standard deviations above control levels of expression was chosen as a cut-off level for increased expression (shaded area). Only human serum and IFN-α2a were capable of inducing an increase >2SD in the proportion of activated DC in culture.

Figure 15 shows the concentration dependent effect of type I IFNs on DC maturation.

Direct comparison of titrations of IFN-α2a (■) (n=3), IFN-α8 (●) (n=1) and IFN-β (▼) (n=1), on antigen expression of DC. DC cultured from a single donor were grown in GM-CSF, TNF-α and IL-4 until day 14, type I IFNs were added daily between day 14 and day 17. FACS analysis was performed on day 17. Cell populations were gated to include only the large cells (20% of total cells).

Figure 16 shows the induction of CD83 expression in DC cultures by IFN-α2a.

CD83 expression of DC cultures without (A) or with (B) IFN-α2a added for 3 days. Progenitor cells were cultured under standard serum-free conditions. On day 14 the culture was split and IFN-α2a (1000 U/ml) was added into one half. FACS analysis was performed on day 17. Vertical gate indicates fluorescence of 98% of cells with control antibody.

Figures 17A and 17B show that the effect of IFN-α on DC between day 13-16 depended on the previous exposure of these cultures to IL-4 (n=3). (GT: GM-CSF (50 ng/mL)+TNF-α (20 ng/mL). GTI: GM-CSF, TNF-α+IL-4 (500 U/mL)).

A) CD80 expression in cultures assessed by flow cytometry on day 16.

B) CD86 expression in cultures assessed by flow cytometry on day 16.

Figure 18 compares the percentage of activated DC in paired cultures with and without 3 days' exposure to IFN-α.

Figure 19 shows the allostimulatory capacity of DC activated by IFN-α in the same six cultures as in Figure 18.

A) The effect of IFN-a2a on the allostimulatory capacity of unsorted cultures of DC was studied after 17 days culture in GM-CSF, TNF-a and IL-4 (■) compared to paired cultures with addition of IFN-a2a (1000 U/ml) days 14-17 (◆), (n=6), $p<0.05$.

B) Allostimulatory function of DC sorted on day 18. Culture was split on day 14 and either continued in GM-CSF, TNF-α and IL-4 (■) or in these three cytokines together with IFN-α2a (1000 U/ml) added on days 14-18 (◆). On day 18, large DC were sorted and the MLR was performed as described in the Materials and Methods.

Figure 20 shows the effect of IFN-a on migration and activation of skin-derived dendritic cells.

A) Cumulative number of migratory skin-derived DC. One representative experiment is shown (n=6). Split skin was cut into paired samples and cultured in serum-free medium with or without IFN-α2a (3000 U/ml). DC migrating out of the skin were counted every 12 hours.

B) CD80 expression of migratory LC after 48 hours: (i) no IFN-α, (ii) with IFN-α. X-axis: fluorescence-height, Y-axis: forward scatter. Gate defines fluorescence with a control antibody.

Detailed Description of the Invention

The invention will now be described in detail by way of reference only to the figures and to the following examples.

Materials and Methods

Media

The serum-free medium X-Vivo 20 was purchased from BioWhittaker, Walkersville MD. Cell lines were grown in RPMI 1640 (Trace Biosciences, Melbourne, Australia) supplemented with 20 mM HEPES, 60 mg/l penicillin G, 12.6 mg/l streptomycin, 2 mM L-glutamine, 1% non-essential amino acids and 10% heat-inactivated FCS (CSL, Melbourne, Australia).

Recombinant Human Cytokines, Antibodies, Peptides

The following cytokines were added to DC cultures: TNF-α (2.5-100 ng/ml) (R&D Systems, Minneapolis, MN); GM-SCF (10-200 ng/ml) (Schering-Plough, Sydney, Australia), SCF (100 ng/ml) (AMGEN, Thousand Oaks, CA). IL-4 (100-1000 U/ml) (Schering-Plough New Jersey, NJ). The following commercial monoclonal antibodies (mAb) were purchased: FITC-conjugated IgG1 isotype control; OKT6, anti-CD1a; PE-conjugated T4, anti-CD4; B3, anti-CD22; IL-2RI, anti-CD25; MY9, anti-CD33; NKH1, anti-CD56; AICD 58, anti-CD58; (Coulter Corp., FL); PE-conjugated IgG1 isotype control; HPCA-2, anti-CD34; (Becton Dickinson, San José, CA); LFA-1, anti-CD11a/18; Kb90, anti-CD11c; PE-conjugated TUK4, anti-CD14; (Dako Corp. Carpenteria, CA); AT 10, anti-CD32/FcγRIII; BB-1, anti-CD80/B7/BB1; (Serotec, Oxford, UK); 5C3, anti-CD40; IT2.2, anti-CD86/B70/B7-2; (PharmIngen, San Diego, CA); FITC-conjugated sheep anti-mouse mAb; Leu11b, anti-CD16; (Silenus, Miami, FL). The following mouse Ab were kindly provided by Dr A. Boyd, The Walter and Eliza Hall Institute, Melbourne: IAG-11 negative control, W6/32, anti-HLA-A,B,C; Ia, anti-HLA-DR; LYM-1, anti-CD2; T3, anti-CD3; T4, anti-CD4; T8, anti-CD8; OKM-1, anti-CD11b; FMC 17, anti-CD14; WMG1, anti-CD15; FMC 63, anti-CD19; B1, anti-CD20, UCHL1, anti-CD45RO; FMC 71, anti-CD45RA; ICAM-1, anti-CD54. The influenza matrix peptide (residues 57-68:

GILGFVFTL) was a gift from Dr. P. Romero, Ludwig Institute for Cancer Research, Lausanne, Switzerland.

Cell Sources, Cell Line

Human bone marrow (BM) and leukapheresis harvest samples were obtained from normal donors and from patients of the Department of Medical Oncology and Clinical Haematology, Royal Melbourne Hospital, Melbourne. Patients with non-Hodgkin's lymphoma or solid tumours received stem cell-mobilizing chemotherapy and granulocyte colony-stimulating factor (G-CSF) as part of their treatment. Rib segments removed during thoracotomy from patients with lung cancer were obtained from the Department of Surgery, Austin Hospital, Melbourne. The human T2 cell line (Hosken and Bevan, 1990) was kindly provided by Dr. P. Romero, Ludwig Institute, Lausanne, Switzerland. Human K562 cells (Lozzio and Lozzio, 1975) were a gift from H. Zogos, Rotary Bone Marrow Research Institute, Melbourne.

Cell Separation and DC Cultures

Mononuclear cells were prepared by density centrifugation on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). Using 14 gauge needles, rib segments were flushed with RPMI to mobilize BM cells. Residual red cells were lysed with red cell lysis buffer (7.79 g/L NH_4Cl, 0.037 g/L EDTA, and 1 g/L $NaHCO_3$) and $CD34^+$ cells were separated with the MACS CD34 progenitor cell isolation kit (Miltenyi Biotec, Sunnyvale, CA) (Lansdorp, 1989). Purity of the $CD34^+$ fraction was assessed by flow cytometry using HPCA-2 mAb (Lansdorp, 1989) and was consistently over 96%. Cells (10^6 ml-4 x 10^6 ml) were cultured in 100 µl X-Vivo 20 with cytokines in 96 well microcultures (Nunc). 50-100% fresh medium and cytokines were added every 2 to 3 days. Proliferating confluent cultures were transferred with an Eppendorf pipette into progressively larger tissue culture plates (48 well plates (Falcon), 24 well plates (Nunc), 12 well plates (Flow Lab. VA) and 6 well plates (Nunc)).

The CD34-fraction was used for HLA typing (Victorian Tissue Typing Service, Royal Melbourne Hospital). The remaining cells were frozen in FCS with 10% DMSO (Merck, Melbourne, Australia) for use as autologous responders or stimulators in later functional assays.

Morphology, Flow Cytometry and FACS

Cytocentrifuge preparations were prepared by applying 10^4 cells to glass slides and spinning for 10 min at 300 rpm (Shandon, Cytospin 2). These were air-dried and stained with May-Grunwald/Giemsa stain.

Cells for flow cytometry were incubated with the primary mAb in blocking buffer (Phosphate buffered saline (PBS) (2.275 g/L Na_2HPO_4 (anhydrous), 0.552 g/L $Na_2HPO_4xH_2O$, 7.0 g/L NaCl in deionized distilled water) with 10% human AB-serum) for 5 min at room temperature (RT), washed in PBS/0.01% Na azide and, where the primary mAb was not directly FITC-or PE-conjugated, incubated with the FITC-labeled secondary mAb. Cells were fixed in PBS/2% Formaldehyde/0.01% Na azide/1% BSA. The immunophenotype was determined using a FACScan flow cytometer (Becton Dickinson). FACS was performed on either a FACStar plus or a modified FACS II flow cytometer.

Mixed Leucocyte Reaction (MLR)

Peripheral blood mononuclear cells (PBMC) of healthy laboratory volunteers (10^5 per 100 µl culture) were used as responders. Cells were cultured for 5 days in RPMI with 10% FCS in 96 well round-bottomed plates. Stimulators were either DC culture populations or frozen and thawed $CD34^-$ cells (60-80% viability) from the same patient. After irradiation (3000 rad), stimulator cells were plated out in triplicate over a range of concentrations (10,000-3,000-1,000-300 per well). After 5 days, cells were incubated with 0.5 µCi/well ^{3}H-thymidine (DuPont, Sydney, MA) for 20 hours, transferred on to a glass fibre filter (Wallac, Turku, Finland), and ^{3}H-thymidine-

incorporation into DNA was measured using an LKB 1205 Betaplate scintillation counter (Wallac).

Mixed Leukocyte Peptide Cultures (MLPC)

The capacity of DC and DC sub-populations to stimulate peptide-specific $CD8^+$ CTL was assessed. DC or autologous PBMC ($CD34^-$ fraction) of $HLA\text{-}A2^+$ patients were pulsed with influenza matrix peptide (10 µg/ml) in the presence of β_2-microglobulin (2.5 µg/ml, Sigma Aldrich, NSW) in X-Vivo 20 for 45 min at 37°C. Stimulators were washed and co-incubated with 10^6 autologous PBMC ($CD34^-$fraction) as responders in X-Vivo 20, 10% pooled human A serum and 10-20 U/ml IL-2 (Pepro Tech, Rocky, NJ). To improve cell-cell interactions during the first week, cultures were established in hanging drops[11] in Terasaki plates (Nunc, 20 µl drops containing approx. 10^5 cells per drop). After 7 days, cultures were transferred into 96 well flat-bottomed plates and re-stimulated with peptide-pulsed, irradiated, autologous PBMC at day 7 and 14. After 3 to 4 weeks, cultures were examined for $CD8^+$ lymphocytes by staining with anti-CD8 mAb, and flow cytometry and ^{51}Cr release assays were performed, depending upon growth and expansion of responder cells.

^{51}Cr Release Assay

When appropriate, responder cells from MLPC were assessed for peptide-specific lysis. T2 cells were labeled with 100 µCi of $Na(^{51}Cr)O_4$ (Du Pont NEN) with 10 µl of W6/32 ascites for 2 hours at 37°C on a rotating stand. After 3 washes in RPMI, cells were incubated with 2.5 µg/ml β_2-microglobulin ± 1 µg/ml peptide in X-Vivo 20 for 1 hour at 37°C, washed once, and re-suspended in RPMI with 10% FCS at 10^4 cells/ml. Responder cells were pre-incubated at different concentrations (from 10^3 to 3 x 10^4 cells/ml) and with 5 x 10^4 K562 for at least 30 min. K562 is a Natural Killer cell (NK)-target and was added to inhibit non-specific lysis by contaminating NK cells. 1,000 ^{51}Cr-

labeled target cells± peptide were added for 4 hours. Control wells containing K562 and labeled target cells ± peptide in medium alone or with 1% Triton-X were used to assess spontaneous release (SR) and maximal release (MR) respectively. Plates were centrifuged (300 rpm, 5 mins) and 75 µl of supernatant was loaded on to a Lumaplate TM containing solid scintillant (Canberra Packard, IL) and air-dried. To calculate peptide-specific lysis, background lysis in wells with unpulsed targets was substracted from lysis of peptide-pulsed targets. Thus, the percentage of peptide-specific lysis at each E:T ratio was calculated as follows:

= % lysis with peptide pulsed targets (pepT)-% lysis with unpulsed targets (T)

$$= \frac{\text{experimental release (pepT)-SR(pepT)}}{\text{MR(pepT)-SR(pepT)}} - \frac{\text{experimental release(T)- SR(T)}}{\text{MR(T)-SR(T)}}$$

Cytokine deprivation and phagocytosis

Bulk DC cultures and sorted DC were washed, then cultured in X-Vivo 20 with 10% human serum for 5 days in order to assess the stability of their phenotype in the absence of exogenous cytokines and their ability to phagocytic *Candida albicans* cells. As a control, adherent PBMC were obtained by density centrifugation as described above from blood samples of normal volunteers and incubated for 1 hour at 37°C in X-Vivo 20 with 20% human serum (HS). Non-adherent cells were removed, and remaining adherent cells incubated in the same medium for another 5 days. An assay to examine the phagocytic ability of DC was performed using heat-killed *Candida albicans*. Cells were washed, cultured in X-Vivo 20 with 10% HS, and *C. albicans* was added for 15 mins at 37°C, 10% CO_2. Phagocytosis was examined in untreated (opsonizing) and heat-inactivated (non-opsonizing) HS over 15 mins, 1 and 2 hours incubation. Cytospins were performed and examined by light microscopy.

Example 1 Characterization of Mature DC Derived from Human $CD34^+$ progenitor Cells Under Serum-Free Conditions

Phenotypic and Morphological Changes of DC in Serum-Free Cultures.

Purified $CD34^+$ progenitor cells were cultured in serum-free medium (X-Vivo 20) in the presence of GM-CSF (10-200 ng/ml) and TNF-α (2.5-100 ng/ml), with and without IL-4 (100-1,000 U/ml). In the absence of serum, cell growth was density-dependent. Since cells failed to grow when seeded at densities lower than 5×10^5 cells/ml (n=4), $CD34^+$ cells were seeded at $>10^6$ cells/ml.

DC development in serum-free conditions was observed as a process of phenotypic and morphological evolution which took 3 to 4 weeks. Figure 1 shows a brief summary of the phenotypic and morphological events which occur during days 14 and 28. At day 14, a proportion (10-25% of bulk culture cells) expresses CD1a, a MHC-like surface antigen known to be expressed on epidermal Langerhans cells. However, DC-associated markers like CD83 and CD86 are only expressed on a minority of the cells and gradually increase until day 28. This process is referred to herein as DC *maturation*. This is illustrated in Figure 1A, which shows the up-regulation of CD83 and CD86 and down-regulation of CD1a on a subpopulation of large cells between days 14 and 28. Figure 1B depicts the corresponding morphological changes. Panel I shows cells at day 14 which included a small percentage of DC which adhered to plastic surfaces (arrows) and were detached here by pipetting. At day 28, most DC were non-adherent and displayed a round phenotype with fine dendrites, as shown in panel II.

Phenotype of Mature DC at day 28

The surface antigen expression pattern of these mature large cells is shown in Figure 2. The cells showed high expression of HLA-A,B,C, HLA-DR, CD40, CD54, CD58 and

CD86, and were positive for CD1a, CD4, CD11a, CD11b, CD11c, CD80, CD83 and CMRF44. They did not express T and B cell markers such as CD2, CD3, CD5, CD8, CD19 and CD20, Fc receptors such as CD16 and CD32, nor monocyte-associated markers such as CD14 and CD45RA. Thus all antigens associated with mature DC (CD80, CD83, CD86, CMRF44) as well as adhesion molecules (CD11a, CD11c, CD54, CD58) are expressed, whereas markers of other lineages are lacking.

Morphologically, they displayed the typical DC features (Mackensen *et al*, 1995) of high forward and side scatter by flow cytometry, a corona of fine dendrites and indented nuclei. As well as the DC, the cultures contained 20 to 80% small cells (Figure 2A). These were negative for CD1a, CD4 and CD86, whereas a subpopulation expressed CD11b and CD15.

Example 2 Function of DC as Antigen-presenting Cells

To evaluate their allostimulatory capacity, DC were tested in MLR and MLPC assays. As shown in Figure 3A, cultures containing DC were far more potent stimulators of allogeneic lymphocytes than PBMC from the same patient (n=10). In order to compare the ability of DC and PBMC to present peptide antigens to $CD8^+$ CTL, MLPC assays were performed using an HLA-A2-restricted peptide (GILGFVFTL) derived from the influenza matrix protein (Figure 3B). As few as 2,800 sorted $CD4^+$ DC were sufficient to stimulate specific cytotoxicity against peptide-pulsed T2 target cells. In contrast, at least 10^5 peptide-pulsed PBMC were required to obtain a measurable effect (n=3). In these experiments, DC were sorted according to cell size to avoid blocking of functional receptors by antibodies (CD1a, CD80, CD86). In order to confirm that peptide-specific lysis was due to $CD8^+$ CTL, effector cells of one MLPC were sorted into $CD8^+$ and $CD8^-$ cells. Only the $CD8^+$ fraction had the ability to lyse peptide-pulsed target cells.

Thus these DC are characterized functionally as non-phagocytic, potent antigen-presenting cells (APC),

stimulating allogeneic lymphocytes in the MLR, and autologous peptide-specific cytotoxic T-lymphocytes in the MLPC.

Example 3 Cytokine deprivation and phagocytosis

The ability of DC to maintain their phenotype when removed from the exogenous cytokine culture environment was tested by culturing the cells in X-Vivo 20 with 10% HS for 5 days. In contrast to adherent PBMC (n=2), mature DC did not transform into adherent macrophages. After 5 days in human serum, DC maintained CD83 expression and did not express monocyte/macrophage associated markers such as CD14 and CD32.

Assessment of their phagocytotic ability showed that DC at days 21 to 28 of culture were able to bind but not phagocytose *C. albicans* (75 cells counted, n=5 experiments after 15 and 40 mins in opsonizing medium, n=2 experiments after 1 h, 2 h, and 5 days in non-opsonizing medium). In contrast, adherent PBMC (2.9 ± 0.3 Candida per cell (n=76 cells counted) and the cells of the small, non-dendritic fraction that were also present in DC cultures (4.5 ± 0.5 Candida per cell, n=92 cells counted) readily phagocytosed the Candida after 40 mins in opsonizing medium.

Example 4 Characterization and Optimization of Serum-Free Culture Conditions

Titration of Cytokines

In the absence of serum, cell growth was density-dependent. Since cells failed to grow when seeded at densities lower than $5x10^5$ cells/mL (n=4), $CD34^+$ cells were seeded at $1-3x10^6$ cells/mL.

To determine conditions for optimal DC generation in serum-free cultures, GM-CSF, TNF-α and/or IL-4 were titrated over a range of concentrations. DC development was quantified using the expression of CD1a at day 14 and of CD86 at day 28.

Day 14 Although not sufficient to stimulate DC growth in the absence of other growth factors, GM-CSF was absolutely required for DC growth (Figure 4A). No significant differences in proportion or yield of $CD1a^+$ cells were observed in a concentration range between 10 and 200 ng/ml GM-CSF. In the presence of GM-CSF (50 ng/ml), TNF-α promoted DC development into $CD1a^+$ cells (Figure 4A). 20 ng/ml TNF-α gave maximum stimulation of CD1a expression, and this concentration was chosen for all further experiments. DC growth was optimal when TNF-α was present for the entire culture period (14 ± 2% $CD1a^+$ cells at day 14, n=5). Addition of TNF-α from day 0 to 5 into cultures containing both GM-CSF and IL-4 resulted in 8 ± 1% (n=4) and from day 10 to 14 in 4 ± 0.7% (n=2) $CD1a^+$ DC. IL-4 further promoted the growth of $CD1a^+$ cells (Figure 4B). At day 14, higher percentages of $CD1a^+$ cells were observed in the presence of IL-4 (no IL-4: 4.8 ± 1%, with IL-4: 14.2 ± 1%, n=5).

Day 28 The highest percentages of $CD86^+$ DC were observed in cultures containing higher concentrations of TNF-α (20-100 ng/ml) in combination with IL-4 (500 U/ml) (Figure 4B). As observed with CD1a expression at day 14, the combined action of all three cytokines (GM-CSF, TNF-α and IL-4) was required for optimal expression of CD86 on DC at day 28 (Figure 4C).

Example 5 Differentiation of $CD14^+$ Macrophages in the Absence of IL-4 and in the Presence of Human Serum From Day 0

If $CD34^+$ cells were cultured in the absence of IL-4, no $CD14^-$, $CD11b^{+++}$, $CD1a^+$ cells developed in significant numbers during the first 2 weeks (not shown).

At day 14 to 16, in the absence of IL-4, cultures contained 26 ± 2.6% (n=16) of medium-sized, $CD14^+$ cells, in addition to a smaller population of large $CD14^-$ cells (5% ± 1%, n=16). After day 14, the $CD14^+$ population gradually reduced, even without adding IL-4 to the cultures

(11.7% ± 3.3% at day 28, n=9). In contrast, the large $CD14^-$ population expanded to 32.3% ± 13.8% of the culture at day 28, n=9 (Figure 5A). The addition of IL-4 from day 0 gave rise to $CD14^-$ DC, even if IL-4 was removed from cultures as early as day 7 (n=3). Late addition (day 10 to 16) of IL-4 to cultures containing $CD14^+$ cells (n=5) or to sorted $CD14^+$ cells (n=2) also decreased CD14 expression (1.4 ± 0.5% $CD14^+$ cells at day 16, n=5).

Interestingly, in the absence of IL-4, a spontaneous reduction of CD14 expression combined with acquisition of a mature DC phenotype was observed between days 14 and 28. This effect did not occur if HS was present from the start in these cultures. We hypothesize that IL-4 is produced in serum-free cultures, leading to the development of mature DC from $CD14^+$ precursors. HS seems to favour the differentiation into $CD14^+$ monocytes/ macrophages if present during the whole culture period. However, mature DC were not inhibited or reverted by HS.

This automaturation of cultures containing $CD14^+$ cells in the absence of IL-4 at day 14 into $CD14^-$, $CD83^+$ DC-containing cultures at day 28 was completely abolished if HS was present from day 0. In the presence of HS all cells expressed CD14 and a monocyte/macrophage phenotype, as shown in Figure 5B. This indicates that HS favours monocyte development, and that serum deprivation may be crucial for DC development *in vitro*.

Example 6 DC Yields in Serum-free Conditions and Effects of Stem Cell Factor (SCF)

The variation in total cell yields with time under serum-free conditions is shown in Figure 6A. Regardless of TNF-α concentration, an early drop in cell numbers followed by a gradual rise was observed. In parallel with this drop in yield there was an increase in the expression of CD33 in the first 3 to 5 days of culture as shown in Figure 6B, indicating that the cultures were

not able to support the survival of non-myeloid cells (n=6).

Different sources of $CD34^+$ cells were compared. These included leukapheresis harvests, BM aspirates and rib fragments from normal donors as well as from cancer patients. The yield of $CD34^+$ cells/ml leukapheresis product was recorded for 19 samples (8 patients). Yields ranged from 2×10^5 to 5×10^7, with a median of 1.9×10^6 $CD34^+$ cells per ml leukapheresis product. Table 1 shows overall cell yields and the proportion of $CD1a^+$DC obtained at day 16 from 12 leukapheresis harvests, 4 rib segments, and 3 normal BM samples, all performed during one 3 month period. In 4 cases, total cell numbers did not exceed the number of seeded $CD34^+$ cells. These results were unpredictable, and the numbers are too small to relate yields to cell source or to the patient's underlying disease (Non-Hodgkin's lymphoma (n=1), Hodgkin's disease (n=1), myelodysplasia/acute myeloid leukaemia (n=1), lung cancer (n=1). Overall, an average of 2.8-fold expansion of total cell numbers was observed. Of these, DC constituted about 20% of cells in the cultures at day 16.

An attempt was made to increase DC yields by including SCF. Addition of SCF (100 ng/ml) from day 0 doubled total cell yields (Table 2) without altering the percentage and phenotype of DC. FCS and human serum (10% from day 0) were likewise able to increase yields (Table 2).

Table 1

Cell Yields After 16 Days of Culture[1]

Sample	Cell number ($x10^{-6}$)	% $CD1a^+$ Cells
Lh^a	1.9	17
Lh^a	2.8	21
Lh^a	1.9	27
LH^a	2.6	20
LH^a	5.5	21
LH^a	5.6	13
LH^a	5.2	20
LH^a	0.8	nd
LH^b	0.7	23
LH^c	3.5	21
LH^d	0.8	nd
LH^e	4.3	14
LH^e	1.9	13
LH^e	3.3	nd
n=14	2.9 + 0.5	19 + 1.3
BM^{rib}	2.6	27
BM^{rib}	3.0	19
BM^{rib}	2.2	22
BM^{rib}	4.4	29
BM^{rib}	4.4	22
BM^{rib}	2.0	22
BM^{rib}	1.3	27
BM^{rib}	3.0	20
BM^{rib}	0.12	24
n=9	2.5 + 0.4	23.5 + 1.1
nBM	5.2	10
nBM	1.9	29
nBM	5.0	26
n=3	4 + 1.1	22 + 5.9

[1] $CD34^+$ HPC were cultured for 16 days in the presence of GM-CSF (10 ng/mL), TNF-α (20 ng/mL) and IL-4 (500 U/mL), at which time cells yields were assessed and CD1a expression evaluated. Yields are calculated based upon a starting inoculum of 10^6 $CD34^+$ HPC. LH (leukapheresis harvest), BM (bone marrow), nBM (normal bone marrow);

[a] Non Hodgkin's Lymphoma (n=8);

[b] Hodgkin's Disease (n=1);

[c] Acute Myeloid Leukaemia (AML) (n=1);

[d] Myelodysplasia-AML (n=1);

[e] Breast Cancer (n=3)

[rib] Lung Cancer (bone marrow of rib fragments)

Table 2

Expansion of Cell Yields[1]

Culture condition	n	Yield (%)
GTI	11	100
GTI+SCF	6	230 ± 33
GTI+FCS (10%)	3	221 ± 73
GTI+HS (10%)	2	620 ± 92

[1] $CD34^+$ HPC were cultured in the presence of GM-CSF (10 ng/mL), TNF-α (20 ng/mL) and IL-4 (500 U/mL) (GTI), with the addition of either SCF (GTI+SCF), Foetal Calf Serum (GTI+FCS) or Human Serum (GTI+HS), for 16 days, at which time cells yields were assessed. Yields are shown as mean±SE relative to cultures without SCF/serum (=100%).

Example 7 Time Course of Differentiation and Maturation of DC from Human CD34$^+$ Progenitor Cells Under Serum-Free Conditions: Complex Phenotypic and Functional Changes

a) DC arise from myeloid precursors

DC development in serum-free conditions was observed as a process of phenotypic and morphological evolution which took 3-4 weeks. The earliest steps of differentiation coincided with the early phase of cell death and resulted in a population of myeloid, CD32$^+$, CD33$^+$, CD34$^+$ progenitor cells around day 3 (Figure 6).

b) DC development between days 3-14

The myeloid progenitors gradually gave rise to a population of large, CD1a$^+$, CD14$^-$, CD32$^-$, CD40$^+$, HLA-DR$^+$ cells. No early peak of CD1a$^+$ precursors was observed around day 6, and no expression of CD14 was found at any time point in the culture (Figure 7). These large, CD1a$^+$ cells were further characterized phenotypically. At day 14, CD1a$^+$ cells expressed low levels of CD4 but no DC activation markers such as CD80, CD83, CD86 or CMRF44 (not shown). They were positive for CD4, CD11b, CD11c, CD13, CD33, CD58 and CD54 (not shown). Between days 3-15, cultures contained a significant proportion of loosely adherent cells with dendritic morphology (Figure 8). It was noted that depletion of non-adherent cells from these cultures removed CD1a$^+$ cells (not shown). At day 14, 3 cultures were sorted into CD1a$^+$ and CD1a$^-$ cells. A significant proportion of CD1a$^+$ DC showed Birbeck Granules (BG) (20-40%) (Figure 8). BG were never seen in CD1a$^-$ cells. At day 14, the CD1a$^-$ cells were a heterogeneous population including phagocytic cells and immature cells (Figure 8C). Small cells were CD1a$^-$, CD14$^-$, CD40$^-$, CD32$^+$, and expressed HLA-DR for a prolonged period (Figure 9A). The expression of HLA-DR was gradually lost as these cells

matured. The myeloid marker CD13 was likewise expressed on all small cells (not shown).

c) Phenotypic changes of maturing DC between day 14 and 28

In serum-free cultures of human $CD34^+$ progenitor cells, two stages of DC differentiation were observed. In the early phase of culture, (10-16 days), a population of large, adherent, Langerhans-like cells developed. These cells were $CD1a^{+++}$, $CD11b^{+++}$, $CD14^-$, $CD80^-$, $CD83^-$, $CD86^-$, HLA-A,B,C^+ and HLA-DR^+ cells (Figure 9A; ———). 20-40% of $CD1a^+$ cells sorted on day 14 contained Birbeck Granules (see Figure 8). During the next 2 weeks (late phase, days 17-28), this population spontaneously matured into non-adherent APC with up-regulation of HLA-A,B,C, HLA-DR, costimulatory molecules (CD80, CD86) and DC lineage associated antigens (CD83, CMRF44). Simultaneously, down regulation of CD11b and CD1a was seen (Figure 9A; ———). The loss of these antigens was slow and was complete between days 28-40. No Birbeck Granules were found in activated DC. This late phase (d14-28), now referred to as phenotypic maturation, was associated with a loss of adherence to plastic (not shown).

d) Asynchronous maturation of intermediate DC between days 14-28

Heterogeneity of expression was found in antigens whose levels changed between days 14-28. This raised the question whether the large sized population at this time point consisted of 2 distinct dendritic and non-dendritic cell populations or whether asynchronous maturation of immature DC temporarily led to the co-existence of immature and mature DC. The experiments shown in Figures 9B and 10 support the second explanation. Figure 9B shows the CD11b expression in one representative culture at three different time points. Most large sized cells expressed high levels of CD11b at day 16. At day 28, 50% of the large cells had down regulated CD11b to low levels, whereas 50% still

expressed high levels of this antigen (cells were gated for size as indicated by the horizontal line and for antigen expression by the dotted line). However, at day 37, more than 80% of the large cells were $CD11b^{low}$. In parallel, a similar percentage of large cells expressed CD86 (not shown). This suggested that the mature phenotype was acquired asynchronously and was still in process at day 28. To confirm this hypothesis and to confirm the assumption that only the large sized population was capable of differentiating into mature DC, cells from these cultures were sorted at day 13 on the basis of CD11b expression and cultured until day 18. Figure 10A shows three different populations sorted at day 13: I=small, $CD11b^-$ cells, II=small $CD11b^+$ cells, III=medium/large $CD11b^{bright}$ cells. Only the $CD11b^{bright}$ cells expressed CD1a (not shown). When cultured for 5 days in the presence of GM-CSF, TNF-α and IL-4 until day 18, 41% of the small $CD11b^-$ population (I) had developed into small, $CD11b^+$ cells by d18 and 19% of the small $CD11b^+$ cells (II) developed into large, $CD11b^{bright}$ cells (Figure 10B). This confirmed a) that DC differentiated asynchronously from progenitor cells, and b) that a proportion of the small cells still represented immature precursors at least until day 14. In contrast, all of the large, $CD11b^{bright}$ population (III) differentiated into non-adherent cells with the phenotype of mature DC ($CD14^-$, $CD86^+$, $CMRF\text{-}44^+$, $CD11b^{-/low}$), (Figure 10B).

The small cell population remaining after 28 days was $HLA\text{-}DR^-$. 50% of these were granulocytes on the basis of CD15 expression.

e) Functional maturation of DC between day 14 and 28

The antigen presenting capacity of cells from unsorted cultures was examined on days 14 and 28. The results are shown in Figures 11 and 12. Direct comparison of cells from both time points was possible because the same donor of responder cells was used within the paired experiments. Figure 11 shows the significantly increased

allostimulatory capacity of day 28 DC cultures compared to day 14 DC cultures. Note that the assays were performed with unsorted cultures containing only 20% DC.

Sorted $CD11b^{low}$ and $CD11b^{bright}/CD4^{+}$ DC were compared in the MLPC. Figure 12 shows that mature $CD11b^{low}$ DC are superior to immature $CD11b^{bright}$ DC in stimulating $CD8^{+}$, peptide-specific CTL.

Our results show that $CD34^{+}$ progenitor cells differentiate in our serum-free system from myeloid progenitors to immature DC and finally into mature DC. The presence of Birbeck Granules and the typical phenotypic changes during DC activation suggest that our culture system favors the development of Langerhans-like DC, similar to epidermal Langerhans cells.

Type I Interferons Enhance the Terminal Activation of Dendritic Cells

Example 8 Serum Factors in Normal Human Serum Enhance CD86 Expression Within 3 Days

The gap between the expression of CD1a (immature DC) and CD86 (mature DC) in the serum-free cultures (Figure 13A) could be closed by the addition of human serum 3 days prior to FACS analysis, as shown in Figure 13B. This led to a search for cytokines which could be involved in this process.

Example 9 Cytokine Effects on the Late Phase of DC Differentiation

To determine whether addition of exogenous cytokines could accelerate DC maturation and to identify those endogenous factors which might be acting, we screened a variety of cytokines for their effects on DC phenotype in serum-free cultures. HLA-DR and/or CD86 up-regulation were used as markers of maturation. For this assay, cytokines were used at a concentration previously found to be active in other systems, as summarised in Table 3.

Table 3

Factors Screened for Activating Activity in DC Culture

Factor tested	Concentration	Source
Interleukin (IL)-1α	10 ng/ml	Hoffmann LaRoche, N.J., gift of Dr. I. Campbell, Dept. of Medicine, Royal Melbourne Hospital (RMH), Melbourne, Australia
IL-1β	100 pg/ml	R&D systems, Minneapolis, MN
IL-2	100 U/ml	PeproTech, New Jersey, NJ
IL-3	100 ng/ml	Dr. Glenn Begley, The Walter and Eliza Hall Institute (WEHI), Melbourne, Australia
IL-4	1000 U/ml	Schering-Plough, Kenilworth, NJ
IL-6	20 ng/ml	Dr. R. Simpson, Ludwig Institute for Cancer $Research (LICR), Melbourne, Australia
IL-7	20 ng/ml	Serotec, Oxford, UK
IL-8	100 ng/ml	Genentech Inc., CA, gift of Dr. I. Campbell, RMH
IL-10	100 U/ml	Schering-Plough, Kenilworth, NJ
IL-12	10 mg/ml	R&D Systems, Minneapolis, MN
IL-13	20 ng/ml	PeproTech EC Ltd., London, UK
GM-CSF	50 ng/ml	Dr. Glenn Begley, WEHI
TNF-α	100 ng/ml	R&D Systems, Minneapolis, MN

TGF-β	1 ng/ml	Dr. S. Chandler, LICR
Interferon (IFN)-α2a	1000 U/ml	Roche Products, DeeWhy, Australia
IFN-γ	1000 U/ml	Boechringer Ingelheim, Germany
PDGF	20 NG/ML	Dr. R. Whitehead, LICR
VEGF	10 ng/ml	Dr. S. Stacker, LICR
IGF-1 (CR3)	50 ng/ml	Dr. R. Whitehead, LICR
IGF-1 (PKC)	50 ng/ml	Dr. R. Whitehead, LICR
LIF	1000 U/ml	Dr. N. Nicola, WEHI
SCF	100 ng/ml	Dr. Glenn Begley, WEHI
FLT-3L	40 ng/ml	Genzyme Corp., Cambridge, MA
LPS: Sterotype 0111:B4	100 ng/ml	Sigma, St. Louis, MO
BSA	1%	Sigma, St. Louis, MO
Human Serum HS-LPS (=LPS removed)	10% 10%	normal donors normal donors; serum filtered with Zetapor filter (Cuno inc. Life Sciences Division, Meriden, CT) to remove LPS.

Cytokines were added on day 14 and cultures were analyzed by flow cytometry for the presence of activated ($HLA\text{-}DR^{bright}$, $CD86^{+}$) DC on day 17. Each set of experiments (n=23) included control cells grown in standard conditions in GM-CSF, TNF-α and IL-4 ("GTI"). The percentage of activated DC in these control cultures on day 17 was referred to as 100%. Standard deviations were calculated for all experiments to control for the variations between individual experiments. The results are summarized in Figure 14, in which twice the mean of all SD was used to define the no-difference interval (grey). Only normal human serum (HS), human serum filtered through a Zetapor filter to remove LPS (HS-LPS) and IFN-α2a were capable of increasing the percentage of activated DC above 2 SD of the control.

Cytokine Effects on the Late Phase of DC Differentiation

If IFN-α was added daily between day 13 and day 16, a significant increase in mature DC was observed (n>10). $CD34^{+}$ progenitor cells were cultured in GM-CSF (40 ng/ml), TNF-α (20 ng/ml) and IL-4 (500 U/ml) for 16 days and labelled with monoclonal antibodies against CD11b and the DC associated markers CD83, CD86 and CMRF-44 prior to sorting. Flow profiles were gated for large sized cells. A second group of cells was cultured under the same conditions, but IFN-α (1000 U/ml) was added daily between days 13 and 16. CD83, CD86 and CMRF44 expression was up-regulated, whereas CD11b expression decreased. Since the down-regulation of antigens might take longer than up-regulation, IFN-α was added in 3 further experiments until day 28. A similar percentage of large sized cells expressed the antigens which up-regulate during DC maturation (CMRF44, CD86). However, down-regulated antigens (CD1a, CD11b) showed far lower expression in the presence of IFN-α. Thus IFN-α accelerated DC maturation.

Example 10 Effects of Type I Interferons on DC Maturation

To determine whether other type I interferons could also accelerate DC maturation, IFN-α2a, IFN-α8 and IFN-β were added to cultures containing GM-CSF, IL-4 and TNF-α. All three interferons had similar capacity to up-regulate HLA-A,B,C, CD80 and CD86 and to down-regulate CD1a and CD11b expression on the large cell population (n=3). These changes occurred within 3 days, and were concentration dependent, in a range of 10-100 U/ml. As shown in Figure 15. Furthermore, up-regulation of HLA-DR, CD83 and CMRF44 expression was observed in response to the IFN-α2a, as shown for CD83 in Figure 16. Cell numbers in culture did not significantly change during the 3 days exposure to IFN-α.

Example 11 IFN-α Activates DC Function

In order to examine whether IFN-α could also activate DC function, the allostimulatory capacity of DC-containing bulk cultures was compared with or without 3 days exposure to IFN-α2a. Figure 18 shows the results of 6 individual experiments. Cultures were split on day 14 and IFN-α2a (1000 U/ml) was added daily for 3 days into one half. Cultures exposed to IFN-α2a contained 23±3.4% $CD86^+$ DC, and these cells showed significantly increased allostimulatory capacity ($*p<0.05$), as shown in Figure 19A. Cultures without IFN-α contained 9±1.5% $CD86^+$ DC on day 17 (Figure 18). These results were confirmed by an MLR using DC sorted on day 18. DC exposed to IFN-α 2a for 3 days were more stimulatory than control DC, as shown in Figure 25B.

Example 12 Effect of TNF-α and IFN-α

In order to investigate whether IFN-α could act alone or required TNF-α to induce DC maturation, cultures were washed on day 14 and continued until day 17 in serum-free medium containing GM-CSF and IL-4 with or without TNF-

α, IFN-α or both. Both TNF-α and IFN-α were required for optimal maturation, as shown in Table 4. Thus, the enhancement of DC activation by IFN-α under serum-free conditions required the presence of TNF-α.

Table 4

Effects of TNF-α and IFN-α on DC Activation Between Days 14 and 17

	DC as % of large cells		
cytokine / Antigen	TNF-α	IFN-α	TNF-α + IFN-α
CD80	54 ± 7	44 ± 7	74 ± 5
CD83	50 ± 2	nd	72 ± 4
CD86	57 ± 10	47 ± 6	84 ± 2

Example 13 Type I Interferon Only Acts in Cultures Supplemented With IL-4

Figures 17A and 17B show the effect of IFN-α addition between days 13-16 on expression of CD80 and CD86 in DC cultures. Cells grown in GM-CSF (40 ng/ml) and TNF-α (20 ng/ml) ("GT") did not up-regulate CD80 or CD86 in response to IFN-α, whereas cells exposed to IL-4 for the whole culture period ("GTI") responded better than cells cultured in GT and IL-4 added only after day 10 (n=3). This is consistent with the results presented in Example 5.

Example 14 IFN-α Production in Serum-Free Cultures of DC

Since type I IFNs were the only cytokines that could stimulate DC maturation, it appeared likely that autocrine or paracrine production of IFN was responsible for DC maturation in our serum-free cultures. This hypothesis was tested by assaying culture supernatants for IFN activity. Supernatants were collected at different time between days 14 and 30. IFN activity corresponding to

12±2 IU/ml (range 8-25 IU/ml) was detected in 10 samples from cultures of 6 patients. In supernatants from cultures of 5 other patients, IFN-like activity was not detectable. These data suggest that type I interferons can be produced by the cells in these cultures, and may act as autocrine or paracrine factors to regulate the final stages of DC maturation.

Since autocrine production of IFN-α can cause significant biological effects even in the absence of detectable activity in the supernatant (Hamilton *et al*, 1996), we regard the presence of measurable activity in cultures from 6 out of 11 patients as significant. The clear effect of exogenously added IFN-α, the low level production of IFN-like activity in the cultures and the prolonged period of immaturity even in the presence of high dose TNF-α all suggest that type I IFNs provide a necessary signal for the induction of DC maturation and activation.

Example 15 IFN-α Activates the Migration of Skin-Derived DC

In order to determine whether this effect was specific for progenitor-derived DC *in vitro*, the effect of IFN-α2a on skin-derived DC was examined. Split skin samples were obtained from the Department of Plastic Surgery, Austin and Repatriation Medical Centre, Melbourne. As shown in Table 5, in the presence of IFN-α (3000 U/ml), increased numbers of migrating dendritic cells were observed compared to those without IFN-α.

Table 5

Effect of IFN-α on Skin-Derived DC Migration

	Dendritic Cells x 10^3	
time / Protocol	24 hours	48 hours
X-Vivo20, no IFN-α	7 ± 2	13 ± 4
X-Vivo20 + IFN-α	44 ± 25	41 ± 13

Figure 20A shows the cumulative DC number of one representative experiment. DC migration was observed in 6 of 10 experiments. Furthermore, migrating DC exposed to IFN-α showed similar CD1a, CD83 and CD86 expression, but accelerated expression of CD80 compared to the cells which migrated in the absence of exogenous IFN-α (Figure 20B).

In addition to the effects of IFN-α on *in vitro* derived DC, we have shown that IFN-α activated migration of resident DC from split skin samples floating in serum-free medium. IFN-a accelerated CD80 expression on these cells similar to its effects on progenitor-derived DC. These results suggest that the adherent, immature DC in our serum-free cultures are similar to skin derived DC in response to type I IFN as well as in phenotype.

DISCUSSION

This study describes a method for growing mature and functionally potent DC from human $CD34^+$ hematopoietic progenitor cells in the absence of serum. Three cytokines reported previously to support DC growth in the presence of serum (GM-CSF, TNF-α and IL-4) were studied.

The development of mature DC appeared to be a multistep process which took 3 to 4 weeks, resulting in >20% cells with typical dendritic morphology and phenotype. Key features were the very high expression of HLA-A,B,C and HLA-DR, expression of adhesion molecules (CD11a, CD11c, CD54, CD58), costimulatory molecules (CD40, CD80, CD86) and

DC-associated molecules (CD83, CMRF44) (Zhou and Tedder, 1995; Hock *et al*, 1994; Fearnley, 1996), and absence of monocyte/macrophage associated markers (CD14, CD32). The expression of Birbeck granules in $CD1a^+$ DC suggests that a Langerhans like cell is developing in these cultures. Although the antigen expression pattern of DC derived from $CD34^+$ cells in serum-free conditions is very similar to that of other DC types (Freudenthal and Steinman, 1990, Nestle *et al*, 1993), CD2 and CD5 are not expressed in our cultures, whereas CD4 was expressed. In contrast, mature DC purified from peripheral blood expressed CD2 and CD5 but lacked CD4 (Zhou and Tedder, 1995; Wood and Freudenthal, 1992; O'Doherty *et al*, 1993; Zhou *et al*, 1992). It is possible that the expression of these markers is dynamic, and reflects the history of cytokine exposure in the life of a DC. However, the alternative hypothesis that T cell markers like CD2 and CD5 might distinguish DC of different lineages has to be considered.

The cells raised in this study represented terminally differentiated, functionally potent DC. In contrast to adherent PBMC, they did not transform into macrophages, but maintained CD83 expression when cytokines were removed. A similar behaviour of committed DC was recently reported in response to M-CSF (Szabolcs *et al*, 1996). Furthermore, these mature DC could be distinguished from macrophages because they were non-phagocytic (Sallusto and Lanzavecchia, 1994), and remained so in the presence of human serum.

Compared to autologous PBMC, DC-cultures were at least 30 times more potent stimulators of allogeneic PBMC proliferation and of the expansion and activation of autologous, peptide-specific CTL. As has been described for cultures containing FCS (Strunk *et al*, 1996), IL-4 had a potent effect on differentiation of DC. We observed that human serum inhibited DC differentiation, which underlines the value of this serum-free culture system obtaining DC under defined conditions. Foetal calf serum which is mainly

used by groups working with progenitor-derived DC contains xenoantigens and the risk of introducing infectious agents make these protocols less desirable for clinical studies. Autologous human serum does not appear to be a better alternative because of its adverse effects on DC differentiation.

As described by others using serum-containing conditions (Szabolcs *et al*, 1995; Caux *et al*, 1992; Santiago *et al*, 1992; Reid *et al*, 1992), GM-CSF and TNF-α were required for DC development under serum-free conditions. Concurring with previous reports (Reid *et al*, 1992), TNF-α was necessary in the early culture period but had an additional effect on optimal DC development beyond the first 5 days of culture. IL-4 had a potent effect on the differentiation pathway of DC, increasing CD1a expression at day 14 and CD86 expression at day 28. When IL-4 was added during the first week and removed after day 7, the cultures already contained precursors committed to a $CD14^-$ lineage that differentiated into $CD83^+$ DC. Addition of IL-4 (after day 10) likewise decreased CD14 expression. This effect of IL-4 on $CD14^+$ cells is well established (Romani *et al*, 1994; Ruppert *et al*, 1991; Romani *et al*, 1990). Interestingly, in the absence of IL-4 a spontaneous reduction of CD14 expression occurred, combined with acquisition of a mature DC phenotype. This effect was suppressed by the addition of HS in the absence of IL-4. A similar spontaneous maturation was previously reported in the presence of FCS, but in this case, bipotential $CD14^+$ cells had to be isolated from bulk cultures before day 6 (Szabolcs *et al*, 1996). It is possible that IL-4 is produced in serum-free cultures, leading to the development of mature DC from $CD14^+$ precursors. The possibility of an IL-4 independent pathway and the effect of early IL-4 addition to $CD14^-$ progenitors and precursors can now be examined.

GM-CSF, TNF-α and IL-4 were necessary for DC maturation, but did not appear to be sufficient for optimal

DC generation under serum-free conditions. Other factors must be important for both proliferation and maturation of these cells. In the presence of serum DC yields are 2-6 fold higher, suggesting that serum contains additional DC growth and/or maturation factor(s). Secondly, a prolonged time course (21-28 days) was required for DC maturation when compared to previous reports, in which DC were generated in serum containing cultures after 6 days (Szabolcs *et al*, 1996). This permissive effect of serum on DC growth in vitro was reported previously (Reid *et al*, 1992). Thirdly, the density dependence of DC production implies that cells in the culture may be providing additional autocrine or paracrine factors to enhance growth.

The time course of increase in cell numbers in serum-free cultures indicates the presence of proliferating $CD33^+$ progenitors in the serum-free cultures. This suggests that a more efficient expansion is possible. The early drop in viable cell number together with the exclusive survival of $CD33^+$ cells suggests a selective cell death of non-myeloid cells.

We investigated the effects of other cytokines on DC yields. As described by others, SCF was capable of expanding total cell yields without interfering with DC development (Hamilton *et al*, 1996). This expansion, however, was less pronounced than that reported in previous studies in the presence of FCS (Hamilton *et al*, 1996). Since SCF is a growth factor with activity which is best seen in synergy with other haemopoietic factors (Molineux *et al*, 1991), our results might be explained by the lack of additional serum factors. This also underlines the potential value of serum-free systems to study interactions of cytokines in defined conditions. Recently, another study was published using serum-free conditions to investigate the effects of cytokines on DC produced from cord blood progenitor cells (Storbl *et al*, 1996). This study demonstrated that TGF-β could protect progenitor cells from

apoptotic cell death (Riedl *et al*, 1997). Studies of TGF-β with $CD34^+$ cells from adults have not been reported and in our hands, TGF-β had only minimal effect on cell yields. This may be due to the different progenitor sources in our study.

Thus it has been shown that it is possible to culture DC from $CD34^+$ progenitor cells under serum-free conditions, and that these cells are potent stimulators of autologous, peptide-specific, $CD8^+$ CTL. This method will be useful to further characterize the growth and differentiation factors involved in DC development (Romani *et al*, 1990) and to study the phenotype of DC precursors. Evidence is presented to show that IL-4 is produced in these cultures and contributes to the maturation of $CD83^+$ DC.

The use of a novel serum-free culture system to produce DC from progenitor cells has enabled us to investigate the cytokines involved in DC maturation and activation. The main findings reported here are the accumulation of an immature DC population around day 14, the prolonged, spontaneous maturation of this immature population into activated DC during the following 14 days, and the capacity of type I interferons to accelerate this maturation within 3 days.

Three different pathways have been described according to their intermediate ("early") stages which result in functional DC of similar phenotype. These include $CD14^+$, monocytic cells (Sallusto, Lanzavecchia, 1994; Sallusto *et al*, 1995), $CD14^-$, $CD1a^+$ Langerhans cells (Frelinger *et al*, 1979; Katz *et al*, 1979) and $CD14^-$, $CD1a^-$ peripheral blood derived DC (Van Voorhis *et al*, 1982). Under the conditions described here, $CD34^+$ cells differentiated to an intermediate stage which is $CD14^-$, $CD1a^+$, and positive for Birbeck Granules. This is consistent with the Langerhans cell phenotype. As with freshly isolated Langerhans cells (Williams *et al*, 1994), these early DC in serum-free cultures were characterized by

intermediate HLA-A,B,C and HLA-DR expression, high expression of CD1a, expression of CD11b and the lack of accessory molecules (CD80, CD86) as well as the DC associated molecules CD83 and CMRF44. In the presence of TNF-α, these immature DC took another 14 days to acquire phenotypic and functional characteristics which are typical for activated DC. The phenotypic and functional changes observed during this process were similar to those seen in other studies (Teunissen *et al*, 1990; Yokozeki *et al*, 1996), and involved the up regulation of HLA-A,B,C, HLA-DR, CD80, CD86, CD83 and CMRF44, down regulation of CD1a and CD11b and functional maturation into highly allostimulatory cells. In parallel, cells lost their ability to adhere to plastic and became non-adherent, round cells with a corona of thin dendrites.

The spontaneous maturation in these serum-free cultures suggested that the production of autocrine or paracrine factors might be involved in this process. We therefore screened a number of cytokines and other molecules for their action in this system. Apart from human serum, only type I IFNs (α or β) were capable of accelerating maturation, so that within 3 days a majority of the large sized cells in culture expressed CD80, CD83, CD86 and started to down regulate CD1a and CD11b. This effect of IFNs added into cultures containing GM-CSF, IL-4 and TNF-α was concentration dependent in a range between 10 and 1000 U/ml, and similar for three different type I IFNs (α2a, α8 and β). In parallel with the phenotypic changes, DC exposed to IFN-α had increased T cell stimulatory capacity. Both IFN-α and TNF-α were required for DC activation between days 14 and 17. Synergy of TNF-α and IFN on HLA class I expression has been previously reported at the level of transcriptional regulation (Johnson and Pober, 1994). The nature of the effector in human serum activating DC remains unknown. It has recently been shown that human serum contains soluble CD14 which forms complexes with LPS and as a complex can activate $CD14^-$ DC

(Verhasselt *et al*, 1997). Therefore, these results also provide an explanation why LPS in the absence of serum did not activate our $CD14^-$ DC.

We have shown that IFN-like activity was produced in most spontaneously maturing serum-free DC cultures. Since autocrine production of IFN-α can cause significant biological effects even in the absence of detectable activity in the supernatant (Hamilton *et al*, 1996), we regard the presence of measurable activity in cultures from 6 out of 11 patients as significant. The clear effect of exogenously added IFN-α, the low level production of IFN-like activity in the cultures, and the prolonged period of immaturity even in the presence of high dose TNF-α all suggest that type I IFNs provide a necessary signal for the induction of DC maturation and activation.

In addition to the effects of IFN-α on in vitro derived DC, we showed that IFN-α activated migration of resident DC from split skin samples floating in serum-free medium. IFN-α accelerated CD80 expression on these cells similar to its effects on progenitor-derived DC. These results suggest that the adherent, immature DC in our serum-free cultures are similar to skin derived DC in response to type I IFN as well as in phenotype.

Our results open a new perspective on the events involved in DC maturation and activation, showing that type I IFN can enhance the effect of TNF-α in the induction of this process. This suggests that in addition to the multiple immunomodulatory effects, type I IFNs may also regulate immune responses at the level of the antigen-presenting cell. Type I interferons have a well established role in the response to infections with viruses (Hertzog *et al*, 1991; van den Broek *et al*, 1995; Hwang *et al*, 1995). Immunomodulatory effects include the promotion of Th1 responses by inhibition of IL-4 and IL-5 secretion (Demeure *et al*, 1994; Belardelli, 1995), increase in IFN-γ producing cells (Brinkmann *et al*, 1993) and effects on IgG production (Romani *et al*, 1989). Our studies show a novel

mechanism, that type I interferons also mediate effects by inducing maturation and activation of DC. This may help to explain the autoimmune phenomena associated with the use of IFN in hepatitis and cancer patients (Hertzog *et al*, 1991; Ronnblom *et al*, 1991; Gisslinger *et al*, 1992; Preziati *et al*, 1995). It may also provide an additional mechanism for the anticancer effects of IFN-α in a variety of tumors (Tsavaris *et al*, 1996; Kirkwood *et al*, 1997; Real *et al*, 1986; Foon, 1997; Sarna *et al*, 1987; Kaido *et al*, 1995).

Importantly, these results may have impact on clinical strategies for developing cancer vaccines. While interferons are being used as adjuvant therapy for cancer and as anti-viral therapy, the effect of interferons on DC function has not previously been defined. IFN-α is therefore a useful candidate as a vaccine adjuvant in clinical trials using tumor antigens as vaccines. Furthermore, this serum-free system should assist the further study of events associated with DC activation as well as providing clinical opportunities using interferon-activated DC as cellular adjuvants.

For clinical applications, IFN-α may be used as an adjuvant to anti-tumour vaccination strategies (*eg.* in conjunction with peptides like MelanA, Tyrosinase, GP100 and the MAGE family, or together with autologous, irradiated tumour cells with and without GM-CSF). The intratumoural injection of IFN-α should be reconsidered, since tumours contain DC which do not induce anti-tumour responses. This may be because intratumoural DC are inhibited from maturation and migration by tumour-produced inhibitors (such as soluble neutralizing IFN-α receptors. Such inhibitors could therefore be blocked by appropriate agents, such as specific antibodies, so as to permit maturation of these DC.

In addition, autoimmune phenomenon are associated with inteferon-α injections. This may likely be caused by activated DC presenting self-antigens. The inhibition of

this pathological condition by inhibiting interferon may therefore be considered.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

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CLAIMS:

1. A method of inducing the maturation of dendritic cells *in vitro*, comprising the step of culturing mononuclear cells in a serum-free medium in the presence of a Type I interferon following an initial phase of growth in the presence of GM-CSF, TNF-α and IL-4.

2. A method according to Claim 1 in which the mononuclear cells are obtained from a mammal which has been pretreated with a cytokine.

3. A method according to Claim 2 in which the mammal has been pretreated with G-CSF, GM-CSF or Flt-3.

4. A method according to any one of Claims 1 to 3 in which the mononuclear cells are $CD34^+$ haematopoietic progenitor cells.

5. A method according to Claim 4 in which the cells which are cultured are peripheral blood progenitor cells or bone marrow progenitor cells.

6. A method according to any one of Claims 1 to 5 in which the cells to be cultured are enriched for $CD34^+$ cells prior to culture.

7. A method according to any one of Claims 1 to 6 in which the Type I interferon is interferon-α, interferon-β, interferon-ω, or consensus interferon.

8. A method according to Claim 7 in which the Type I interferon is interferon-α2a, interferon-α8 or interferon-β.

9. A method according to any one of Claims 1 to 8 in which the culture medium additionally comprises stem cell factor (SCF).

10. A method according to any one of Claims 1 to 9 in which the cells are cultured for 14 to 35 days.

11. A method according to Claim 10 in which the cells are cultured for 14 to 28 days.

12. A method according to Claim 11 in which the cells are cultured for 14 to 17 days.

13. A method according to any one of Claims 1 to 12 in which the GM-CSF and TNF-α are present in the culture

medium during the whole period of culture, and the IL-4 is present commencing at day 6 or is present for the whole period of culture.

14. A method according to any one of Claims 1 to 8 in which the Type I interferon is present in the culture medium commencing at day 13 of culture.

15. A method according to any one of Claims 4 to 9 in which the SCF is present in the culture medium for the whole period of culture.

16. A method of enhancing the antigen-presenting capacity of dendritic cells, comprising the step of exposing dendritic cells to a Type I interferon.

17. A method according to Claim 16, in which interferon is administered to a recipient mammal *in vivo*, in order to activate the recipient's own resident dendritic cells, including Langerhans cells or other tissue antigen-presenting cells.

18. A method according to Claim 16, in which antigen-presenting cells are infused or injected into the recipient, and interferon is sequentially or simultaneously administered systemically to the recipient mammal.

19. A method according to Claim 17 or Claim 18 in which additional cytokines which induce proliferation of dendritic cells are administered sequentially or simultaneously to the recipient mammal.

20. A method according to Claim 19 in which interferon is given sequentially or simultaneously with GM-CSF or GM-CSF and IL-4.

21. A method according to any one of Claims 16 to 20 in which the Type I interferon is interferon-α, interferon-β, interferon-ω, or consensus interferon.

22. A method according to any one of Claims 16 to 20 in which the Type I interferon is interferon-α2a, interferon-α8 or interferon-β.

23. A method according to Claim 16 in which dendritic cells are subjected to maturation by the method of any one of Claims 1 to 15.

24. A method of preparation of a cellular adjuvant for treatment of a pathological condition, comprising the steps of maturing dendritic cells *in vitro* according to the method of any one of Claims 1 to 15, and exposing the matured dendritic cells to an antigen associated with said condition.

25. A method according to Claim 24 in which the condition is a neoplastic disease and the antigen is derived from neoplastic cells.

26. A method according to Claim 24 in which the condition is a disease caused by an infectious agent and the antigen is derived from the infectious agent.

27. A method according to Claim 26 in which the infectious agent is a bacterium, a virus, a yeast or a parasite.

28. A method according to any one of Claims 24 to 27 in which the antigen is a protein, peptide, polysaccharide, or nucleic acid.

29. A method according to any one of Claims 24 to 28 in which the dendritic cells are transfected with the antigen.

30. A method according to any one of Claims 24 to 28 in which the dendritic cells are incubated *in vitro* with the antigen.

31. A method of producing activated T cells, comprising the steps of harvesting dendritic cells and lymphocytes from peripheral blood or bone marrow of a patient suffering from a tumour, maturing dendritic cells according to the method of any one of Claims 1 to 15, and culturing the dendritic cells and the lymphocytes, either separately or together, in the presence of an antigen, and optionally in the presence of a cytokine.

32. A method according to Claim 31 in which the antigen is a tumour-associated antigen.

33. A method according to Claim 31 or Claim 32 in which the cytokine is a Type I interferon.

34. A method according to Claim 33 in which the interferon is interferon-α, interferon-β, interferon-ω or consensus interferon.

35. A method according to Claim 33 or Claim 34 in which the interferon-α is interferon-α2a or interferon-α8 or interferon-β.

36. A population of mature dendritic cells which:

(a) are non-phagocytic;

(b) have potent antigen-presenting activity;

(c) are capable of stimulating multiplication of allogeneic lymphocytes in the mixed leukocyte reaction (MLR),

(d) are capable of stimulating autologous, peptide-specific cytotoxic T lymphocytes in the mixed leukocyte peptide culture assay system (MLPC), and

(e) which are produced under serum-free conditions.

37. A population of mature dendritic cells according to Claim 36 which strongly express MHC Class I and Class II antigens, CD80, CD83, CD86, and CMR44, and which either express CD1a and CD11b weakly or do not express CD1a and CD11b.

38. Dendritic cell-activated tumour-associated T cells produced by the method of Claim 32.

39. A cellular adjuvant produced by the method of any one of Claims 24 to 30.

40. A method of treatment of a pathological condition, comprising the step of administering an effective dose of the cellular adjuvant according to Claim 39 to a mammal in need of such treatment.

41. A method of treatment of a pathological condition, comprising the step of administering an effective dose of mature dendritic cells according to Claim 36 or Claim 37 to a mammal in need of such treatment, together with or subsequently to administration of an antigen associated with the condition.

42. A method according to Claim 41 in which the condition is a neoplastic disease and the antigen is derived from neoplastic cells.

43. A method according to Claim 41 in which the condition is an infectious condition.

44. A method according to Claim 43 in which the condition is a disease caused by an infectious agent and the antigen is derived from the infectious agent.

45. A method according to any one of Claims 41 to 44 in which the antigen is a protein, peptide, polysaccharide or nucleic acid.

46. A method according to any one of Claims 41 to 45 in which the dendritic cells are the mammal's own cells.

47. A method according to any one of Claims 42 to 46 in which the neoplastic cell antigen is from the mammal's own tumour cells.

48. A method of treatment of a mammal suffering from a tumour, comprising the step of administering an effective dose of dendritic cell-activated, tumour-associated T cells according to Claim 38 to a mammal in need of such treatment.

49. A method according to any one of Claims 42 to 48, in which the tumour antigen is a cancer-associated peptide such as Melan A, tyrosinase, GP100, an antigen of the MAGE family, a cancer testis antigen (CT) detectable by the SEREX method, or a member of the family of cancer antigens.

50. A method according to any one of Claims 42 to 48, in which the antigen is derived from cancer cells or is autologous, irradiated tumour cells.

51. A method according to any one of Claims 42 to 50 in which the antigen is administered in conjunction with GM-CSF.

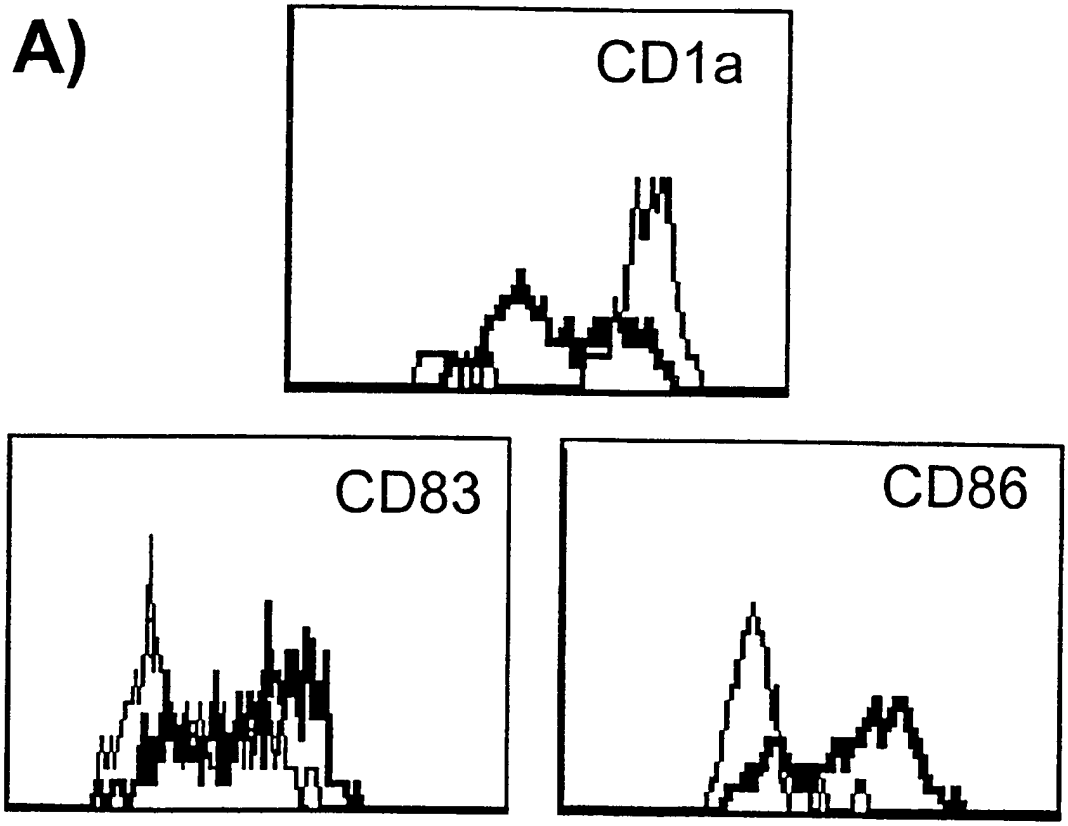
52. A method of using a Type I interferon as an adjuvant for peptide-based anti-tumour vaccination, comprising the step of administering a Type I interferon locally or systemically to a patient in need of such treatment, at the same time as or 1 to 3 days after

vaccination of the patient with a tumour peptide or tumour cell lysate, optionally in conjunction with GM-CSF.

53. A method of using a type I interferon as an adjuvant for vaccination against an infectious agent, comprising the step of administering a Type I interferon locally or systemically to a patient in need of such treatment, at the same time as or 1 to 3 days after vaccination of the patient with the vaccine, optionally in conjunction with GM-CSF.

54. A method according to Claim 53 in which the infectious agent is a bacterium, a virus, a yeast or a parasite.

A)



B)

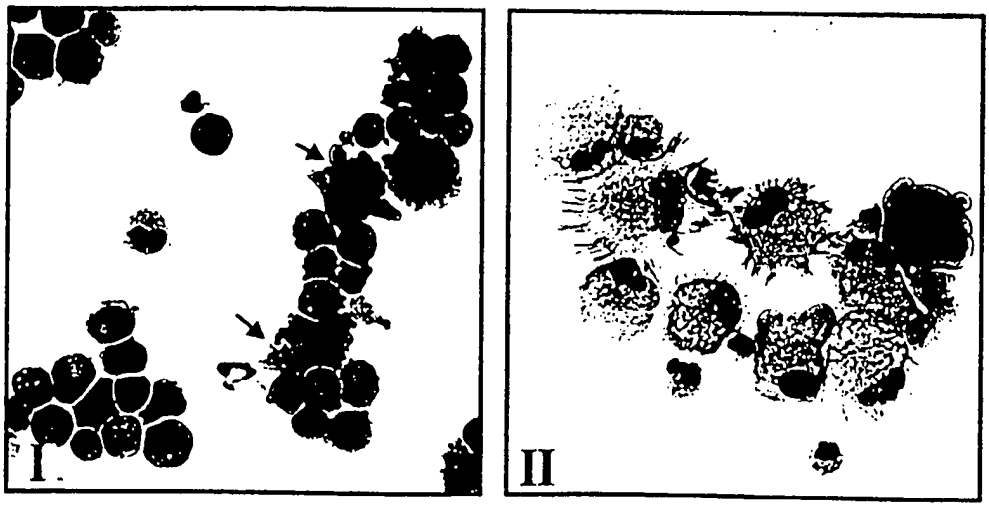


FIGURE 1

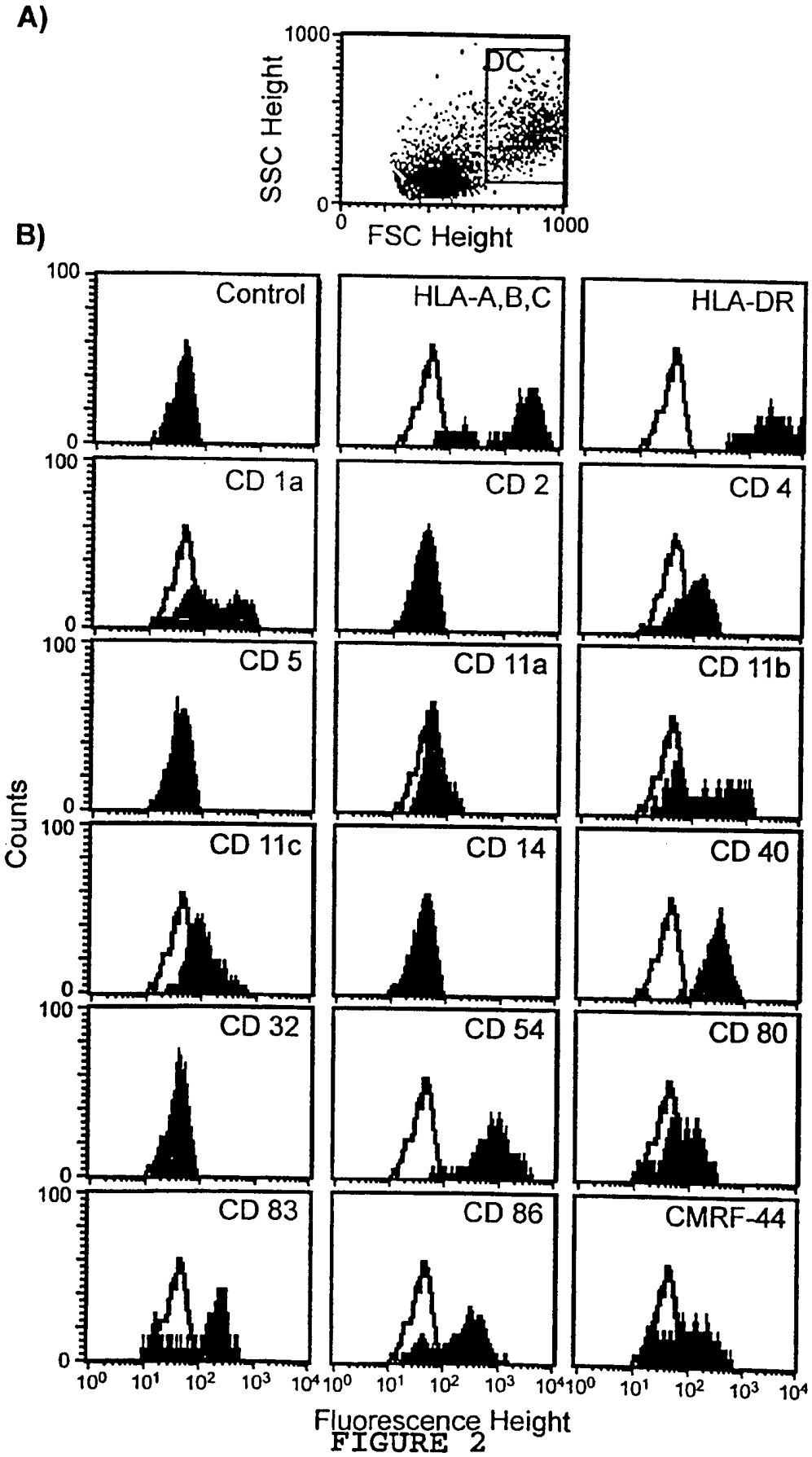
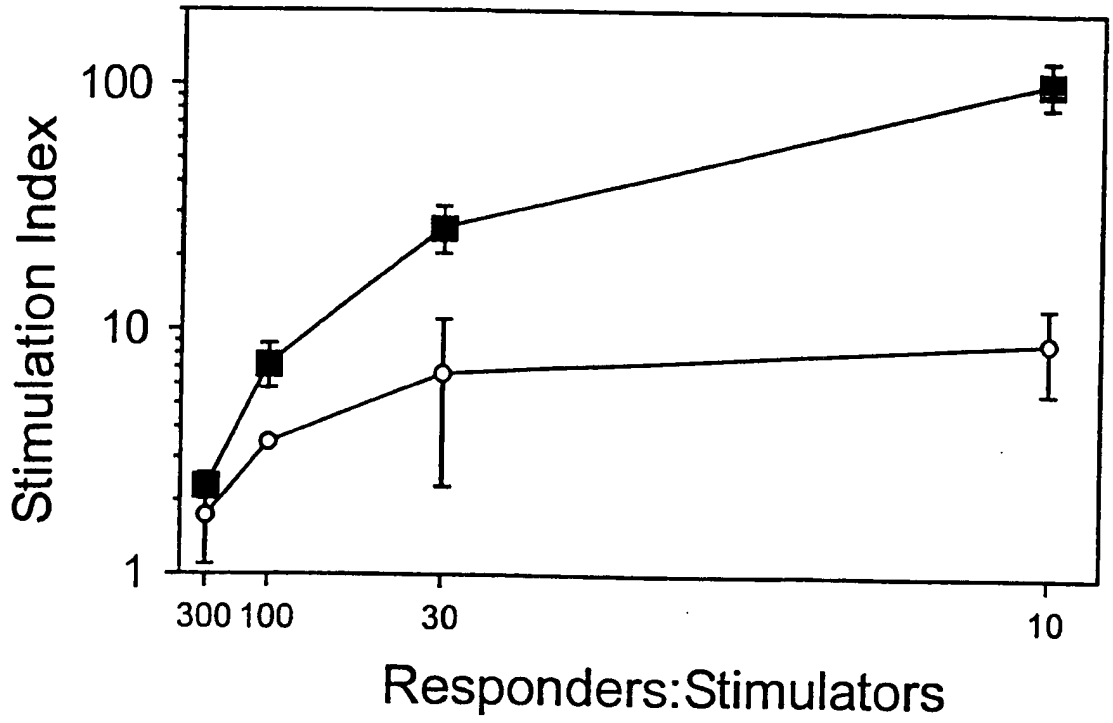

A)
1000
SSC Height
0
DC
0
FSC Height
1000
B)
100
Control
HLA-A,B,C
HLA-DR
0
CD 1a
CD 2
CD 4
CD 5
CD 11a
CD 11b
Counts
CD 11c
CD 14
CD 40
CD 32
CD 54
CD 80
CD 83
CD 86
CMRF-44
10^0 10^1 10^2 10^3 10^4
Fluorescence Height

FIGURE 2


Stimulation Index
100
10
1
300 100
30
10
Responders:Stimulators

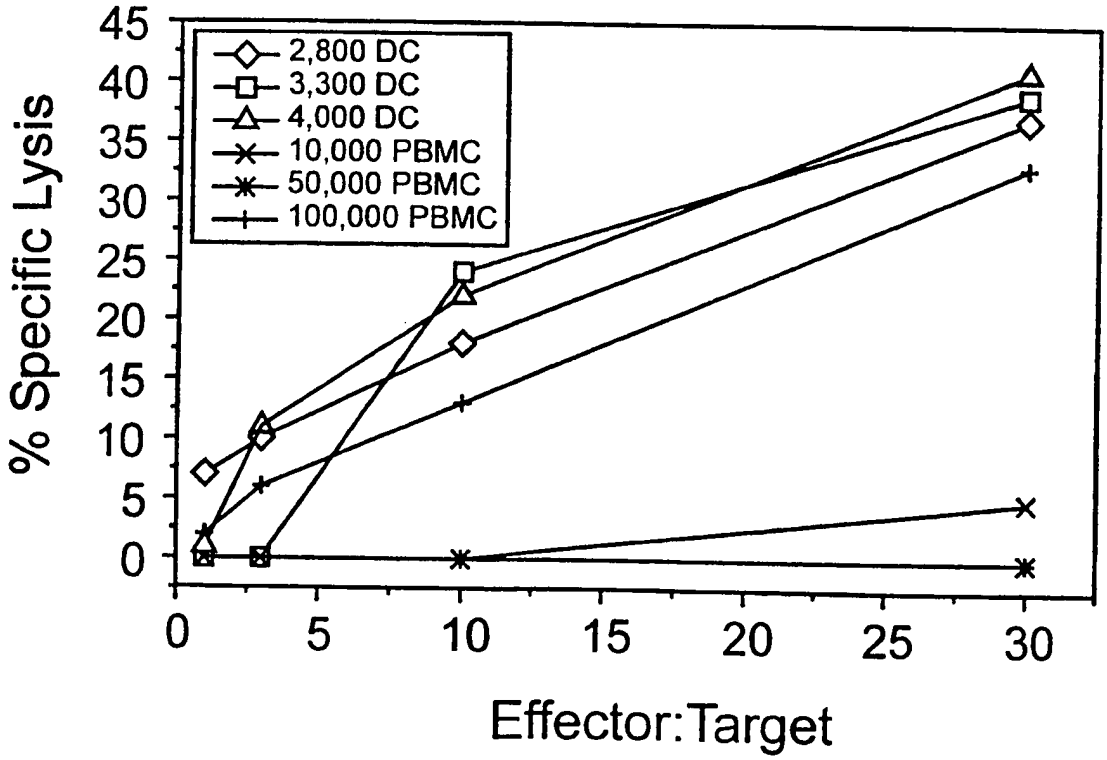

% Specific Lysis
45
40
35
30
25
20
15
10
5
0
2,800 DC
3,300 DC
4,000 DC
10,000 PBMC
50,000 PBMC
100,000 PBMC
0
5
10
15
20
25
30
Effector:Target

FIGURE 3

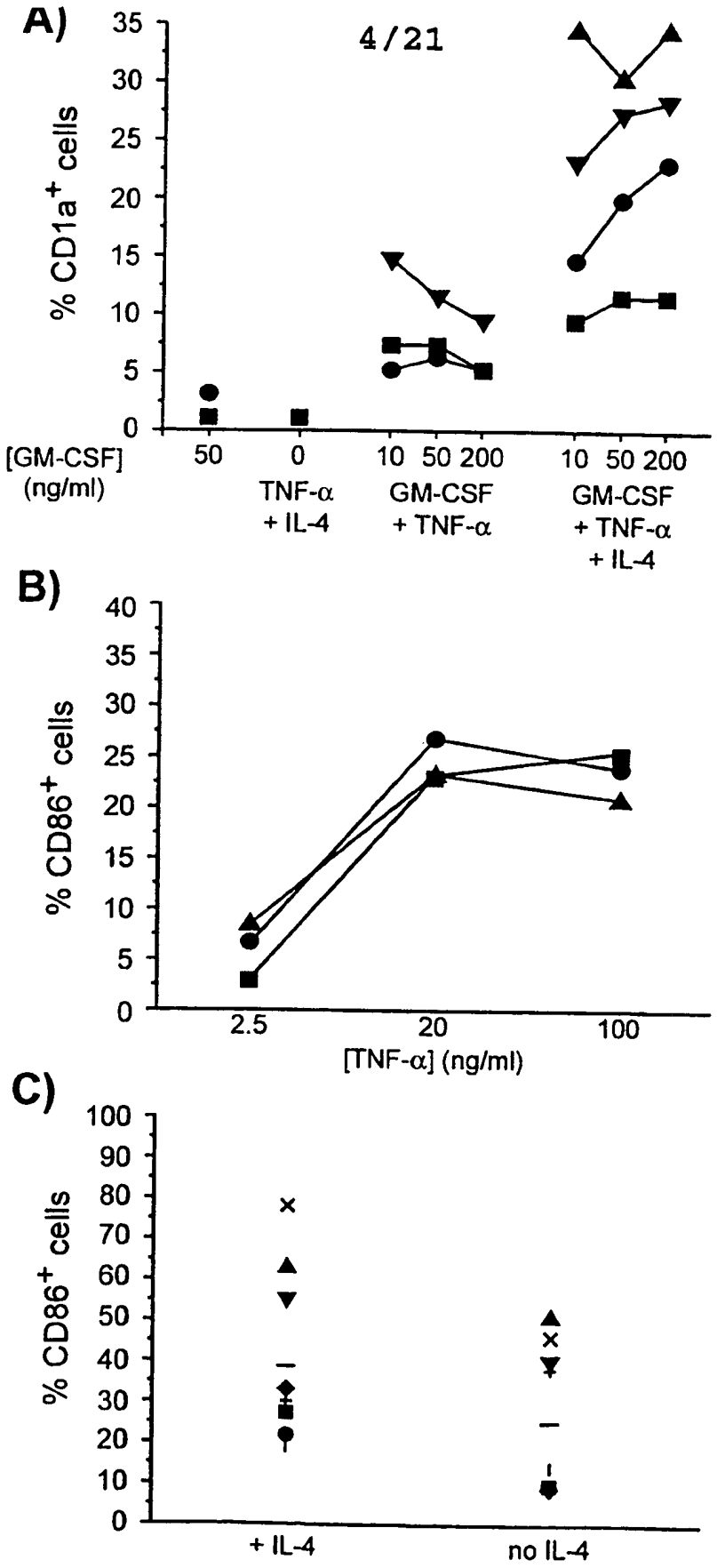

A)
4/21
% CD1a+ cells
35
30
25
20
15
10
5
0
[GM-CSF] (ng/ml)
50
0
TNF-α + IL-4
10 50 200
GM-CSF + TNF-α
10 50 200
GM-CSF + TNF-α + IL-4
B)
% CD86+ cells
40
35
30
25
20
15
10
5
0
2.5
20
100
[TNF-α] (ng/ml)
C)
% CD86+ cells
100
90
80
70
60
50
40
30
20
10
0
+ IL-4
no IL-4

FIGURE 4

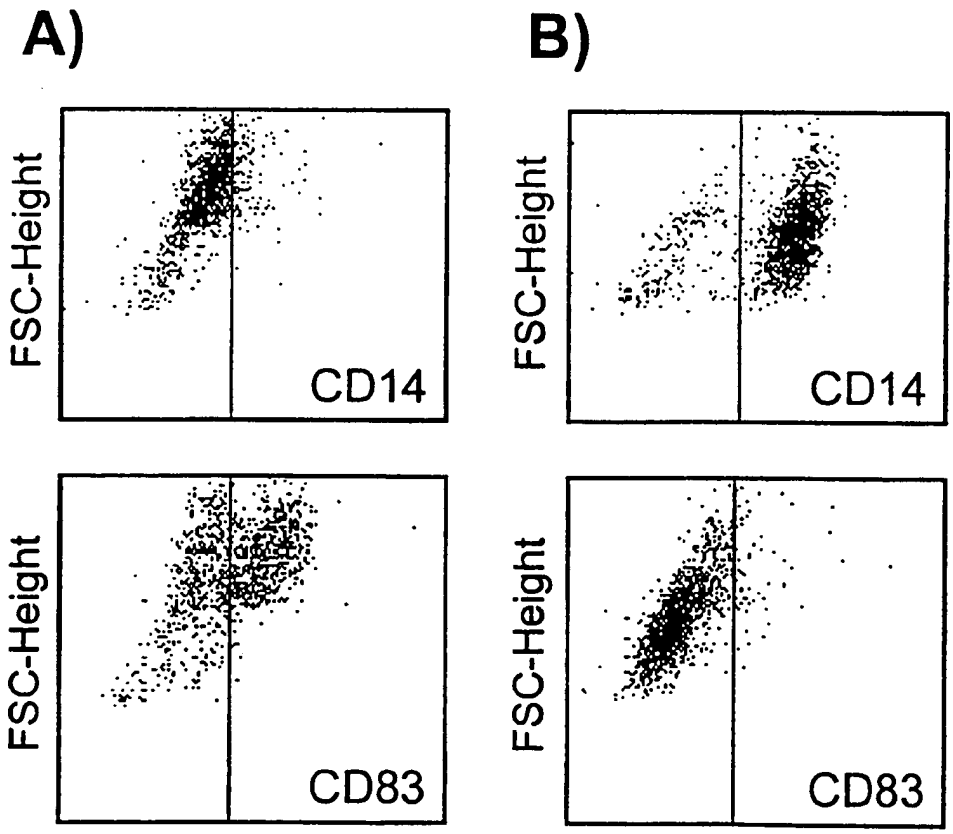

A)
B)
FSC-Height
CD14
FSC-Height
CD14
FSC-Height
CD83
FSC-Height
CD83

FIGURE 5

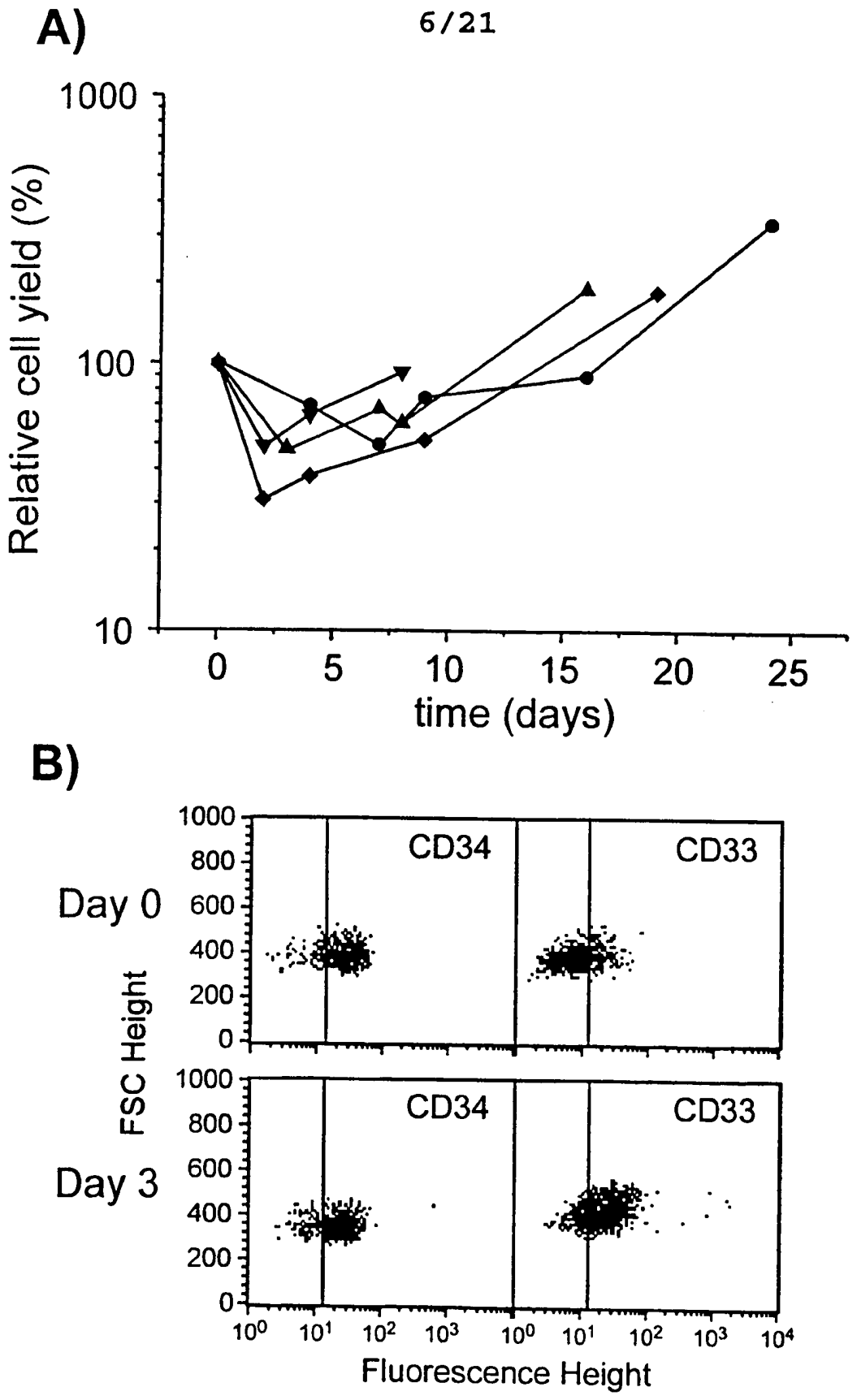

A)
1000
100
10
Relative cell yield (%)
0
5
10
15
20
25
time (days)
B)
Day 0
Day 3
FSC Height
CD34
CD33
1000
800
600
400
200
0
10^0
10^1
10^2
10^3
10^4
Fluorescence Height

FIGURE 6

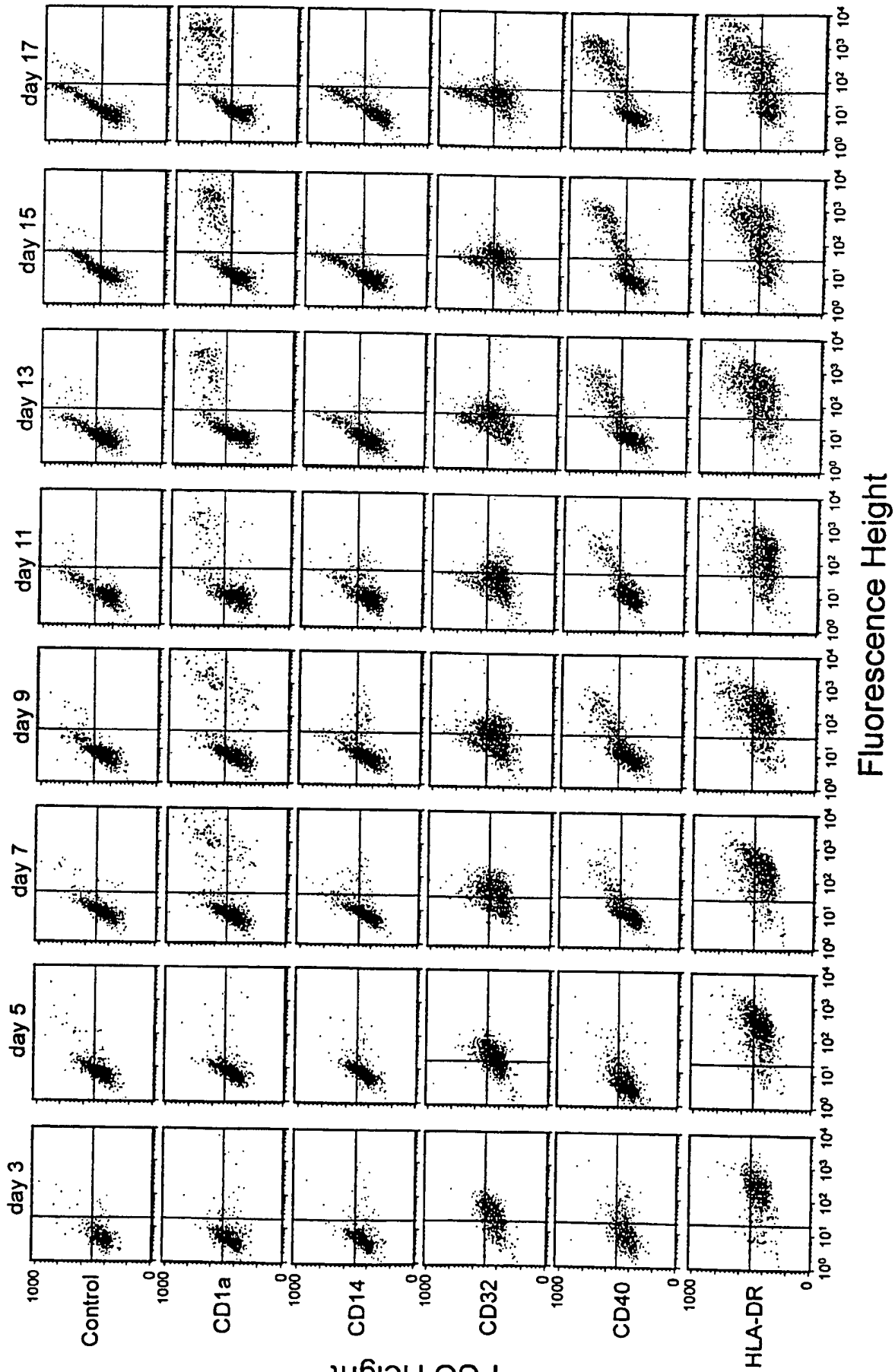

day 3
day 5
day 7
day 9
day 11
day 13
day 15
day 17
Control
CD1a
CD14
CD32
CD40
HLA-DR
1000
0
FSC Height
Fluorescence Height

FIGURE 7

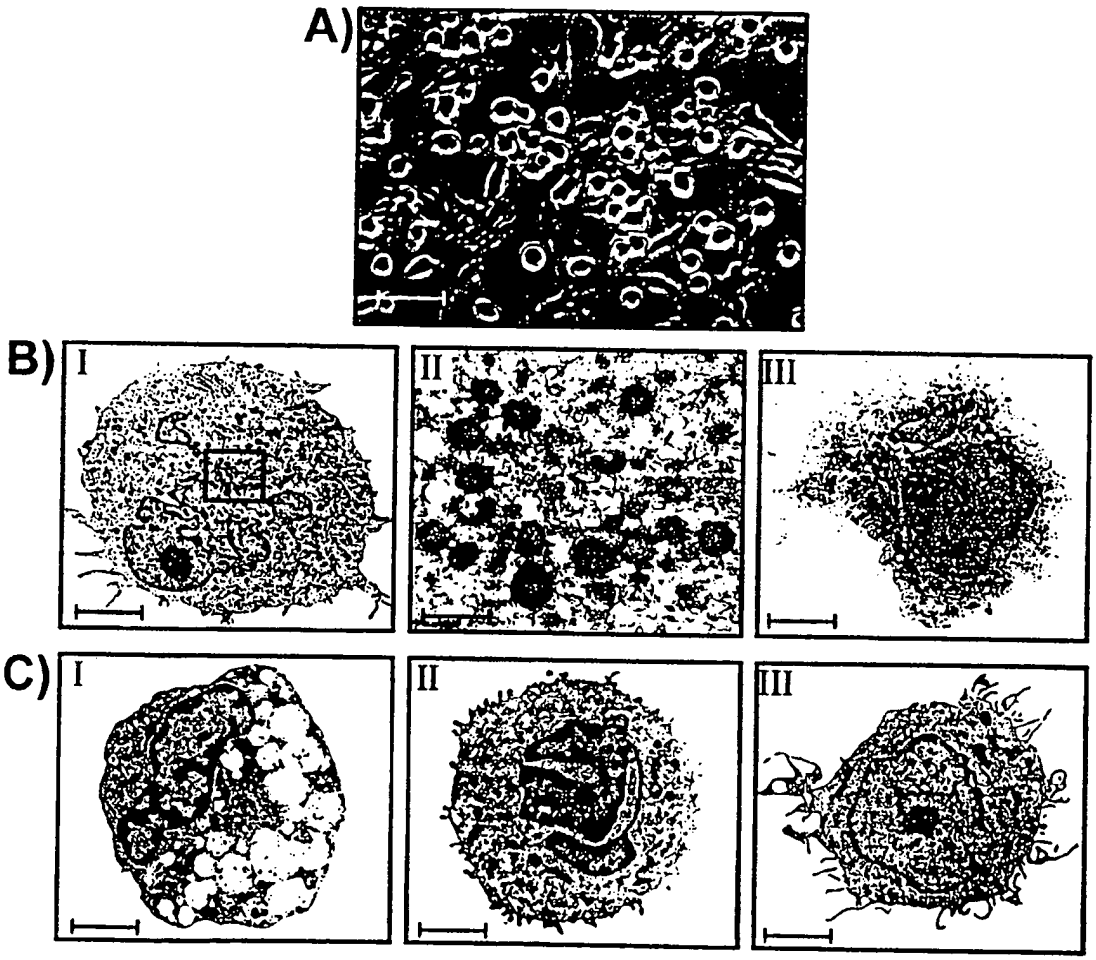


FIGURE 8

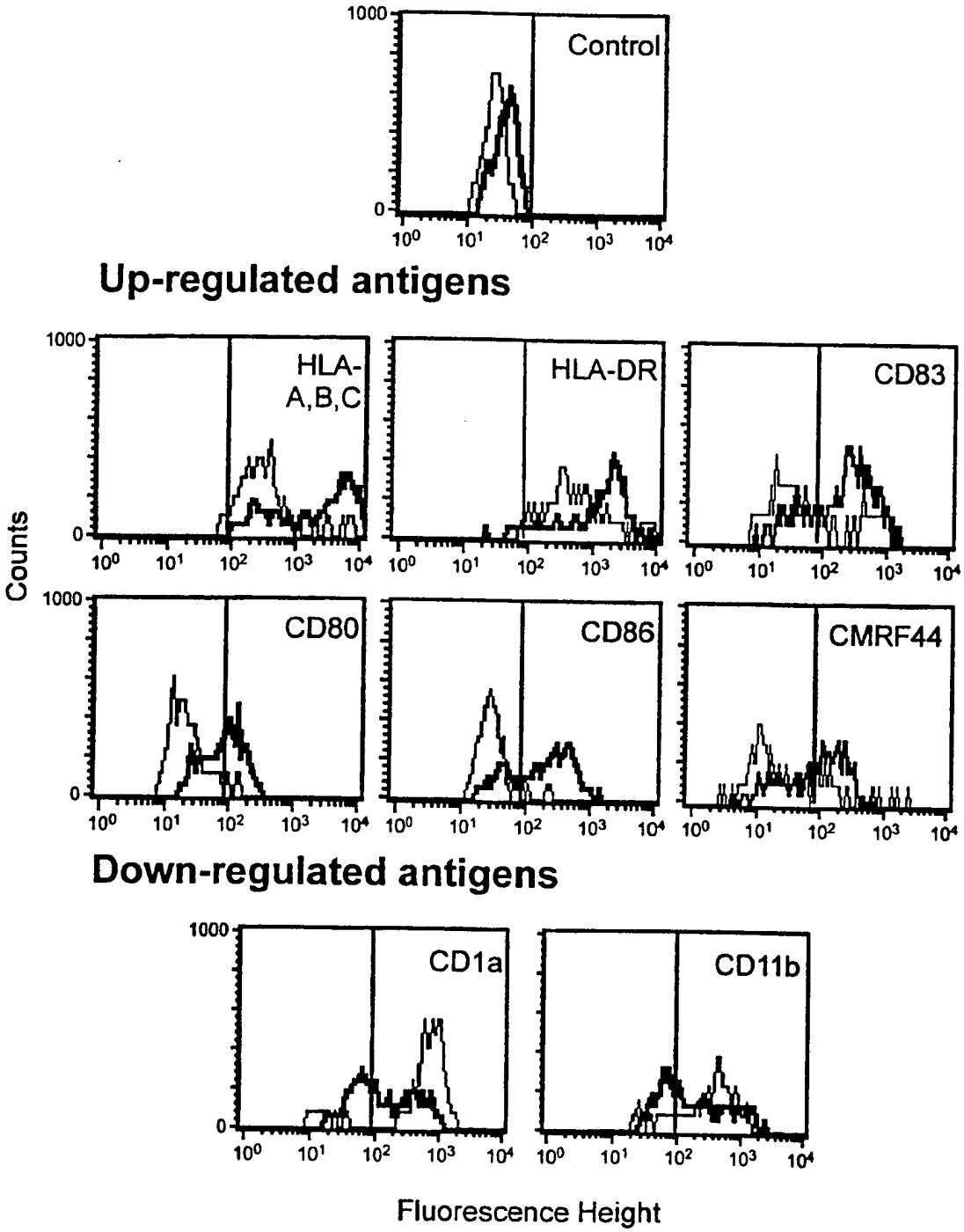

Control
Up-regulated antigens
HLA-A,B,C
HLA-DR
CD83
CD80
CD86
CMRF44
Counts
Down-regulated antigens
CD1a
CD11b
Fluorescence Height

FIGURE 9A

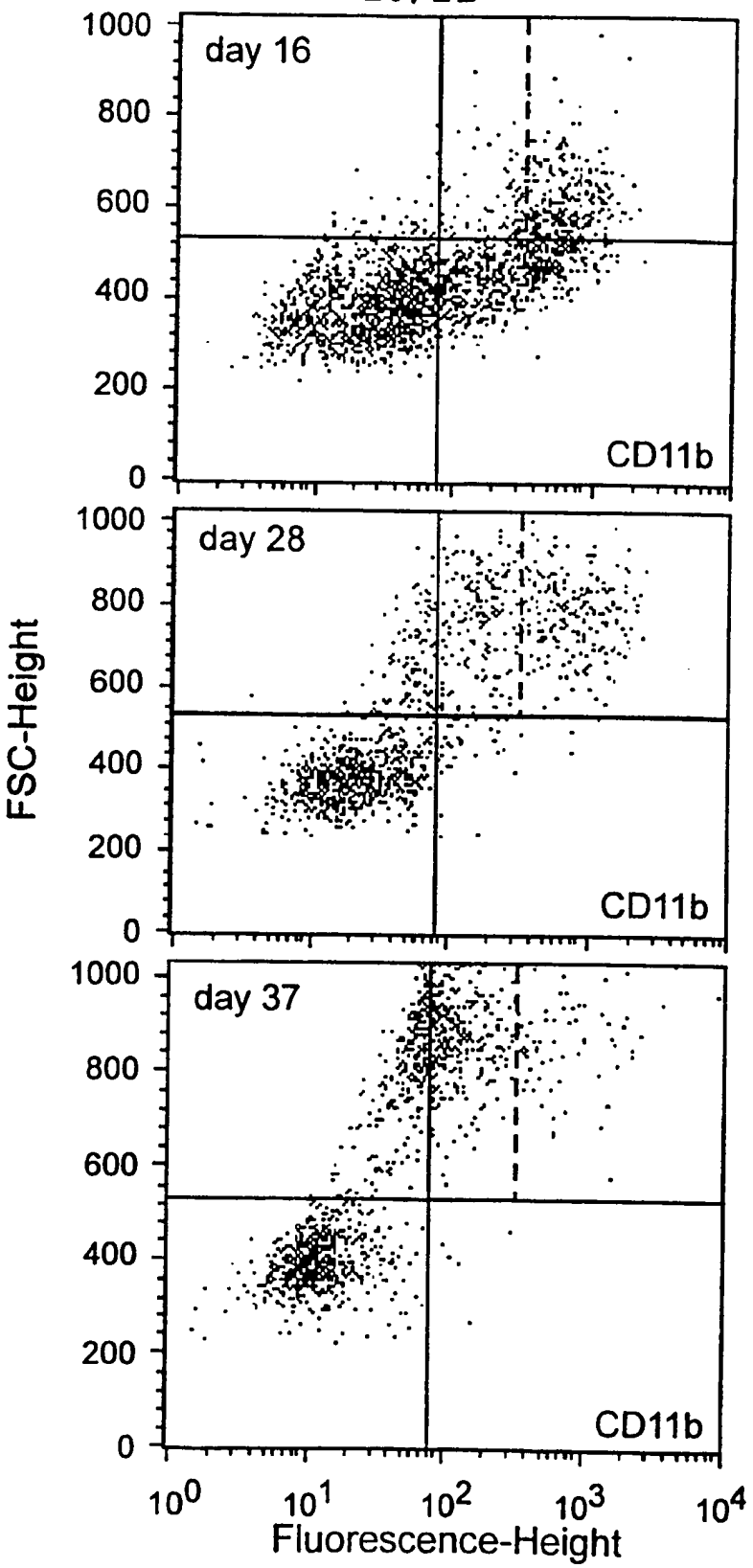

day 16
day 28
day 37
CD11b
CD11b
CD11b
FSC-Height
Fluorescence-Height
0
200
400
600
800
1000
10^0
10^1
10^2
10^3
10^4

FIGURE 9B

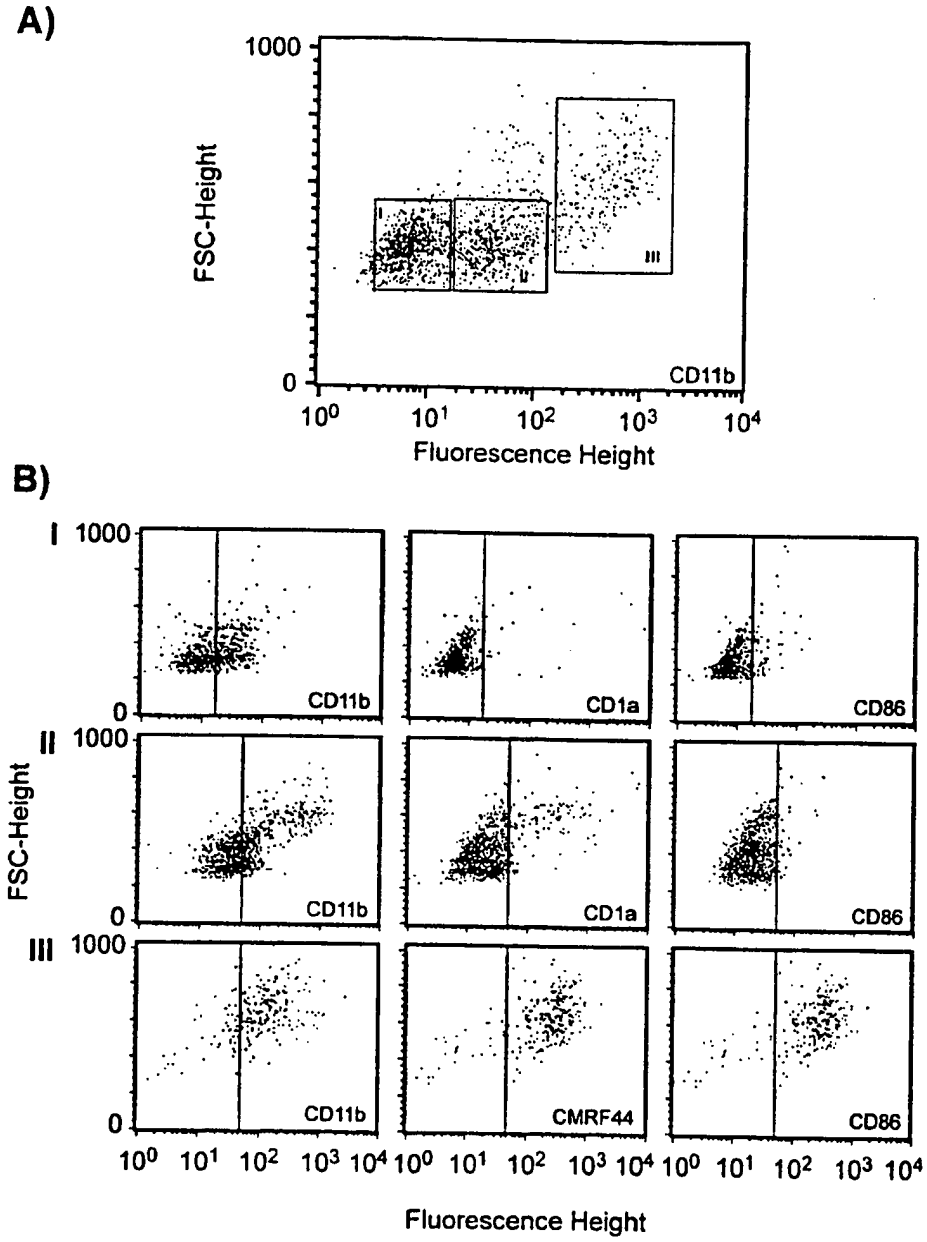

A)
1000
0
FSC-Height
CD11b
10^0 10^1 10^2 10^3 10^4
Fluorescence Height
B)
I
II
III
CD11b
CD1a
CD86
CMRF44
FSC-Height
Fluorescence Height

FIGURE 10

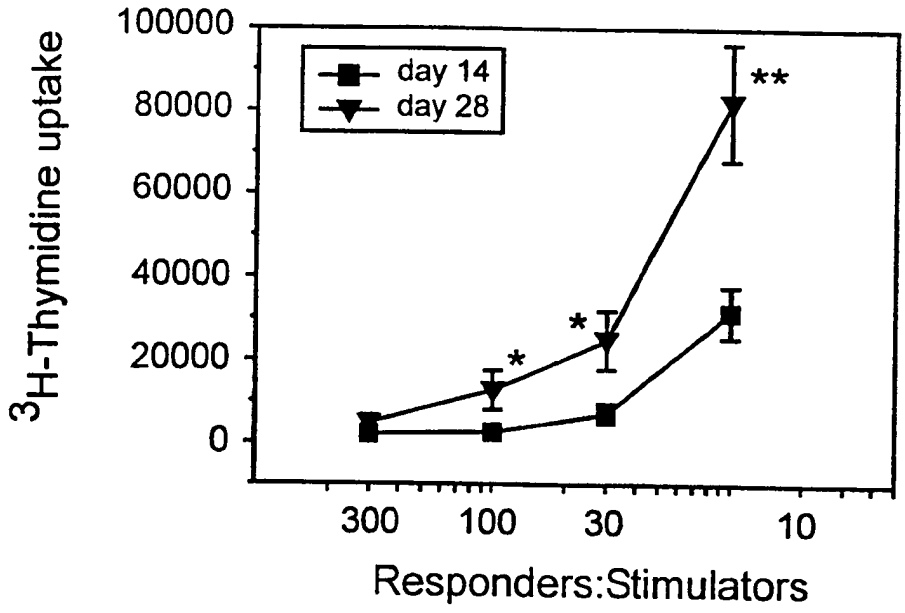

100000
80000
60000
40000
20000
0
^{3}H-Thymidine uptake
day 14
day 28
*
*
**
300
100
30
10
Responders:Stimulators

FIGURE 11

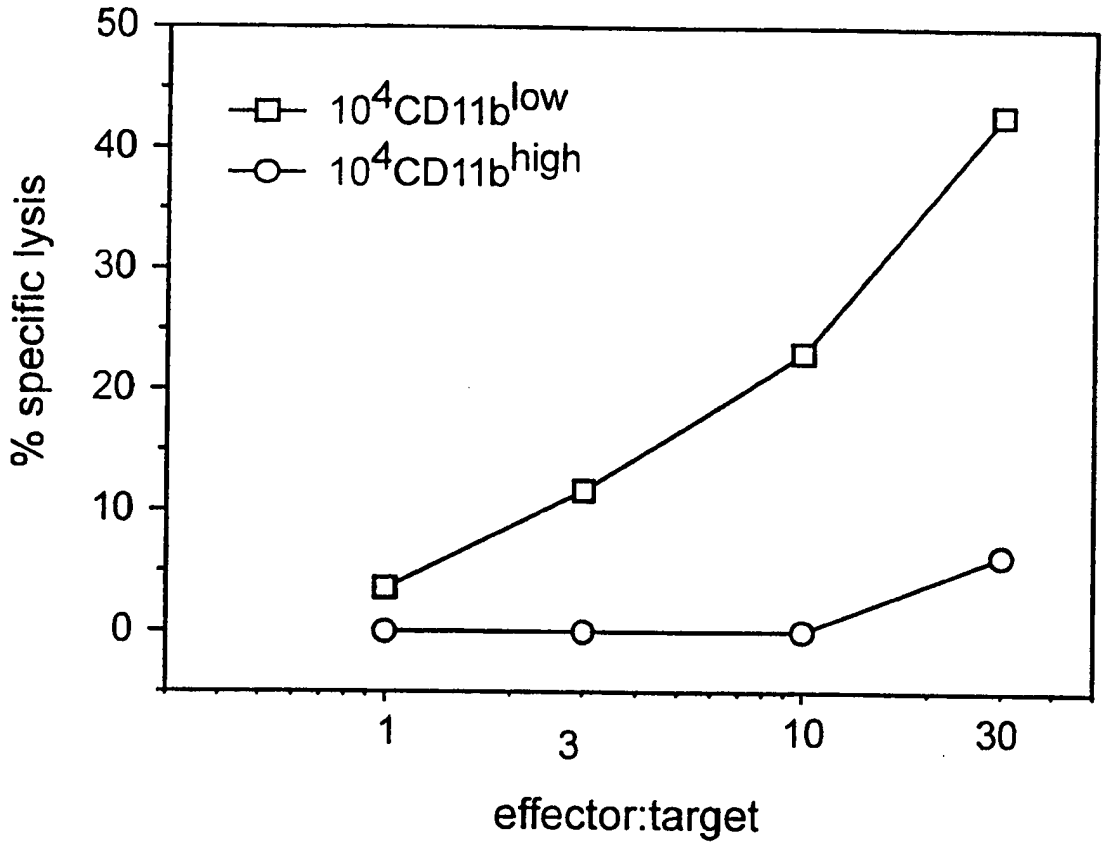
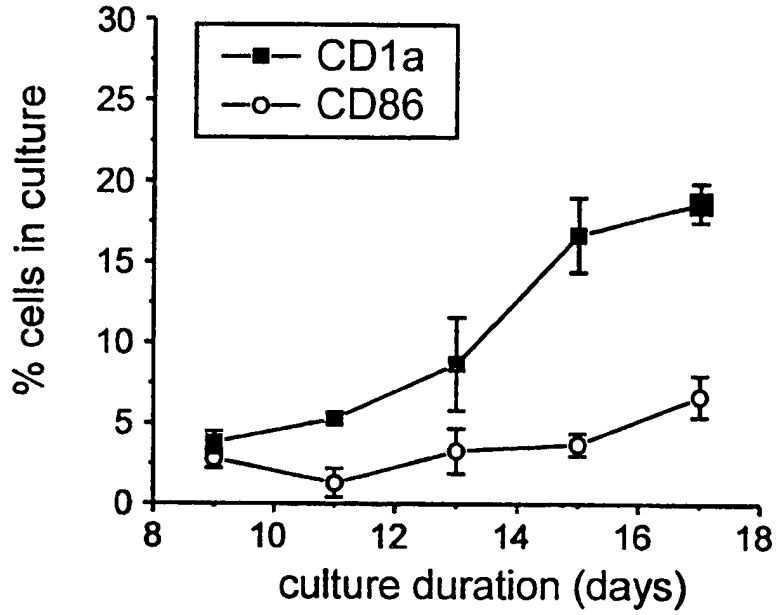

50
40
30
20
10
0
% specific lysis
10^4CD11b^{low}
10^4CD11b^{high}
1
3
10
30
effector:target

FIGURE 12

A) No human serum



B) + human serum

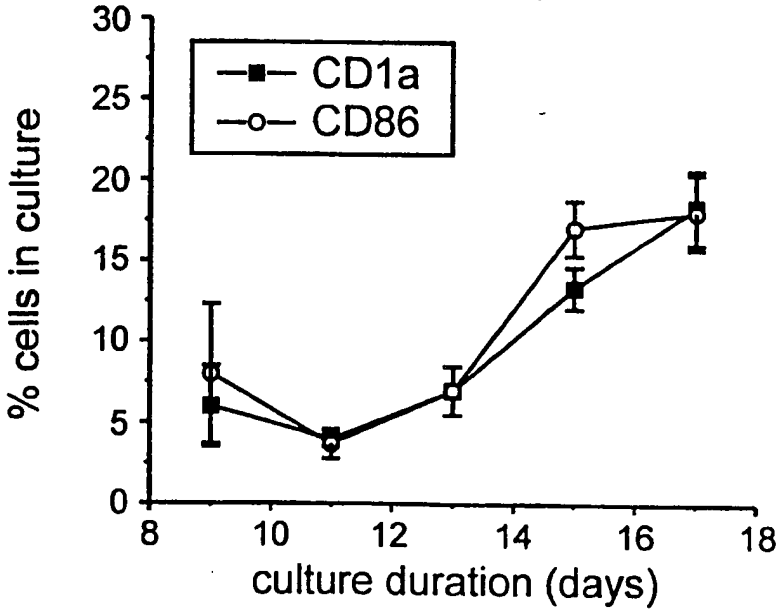


FIGURE 13

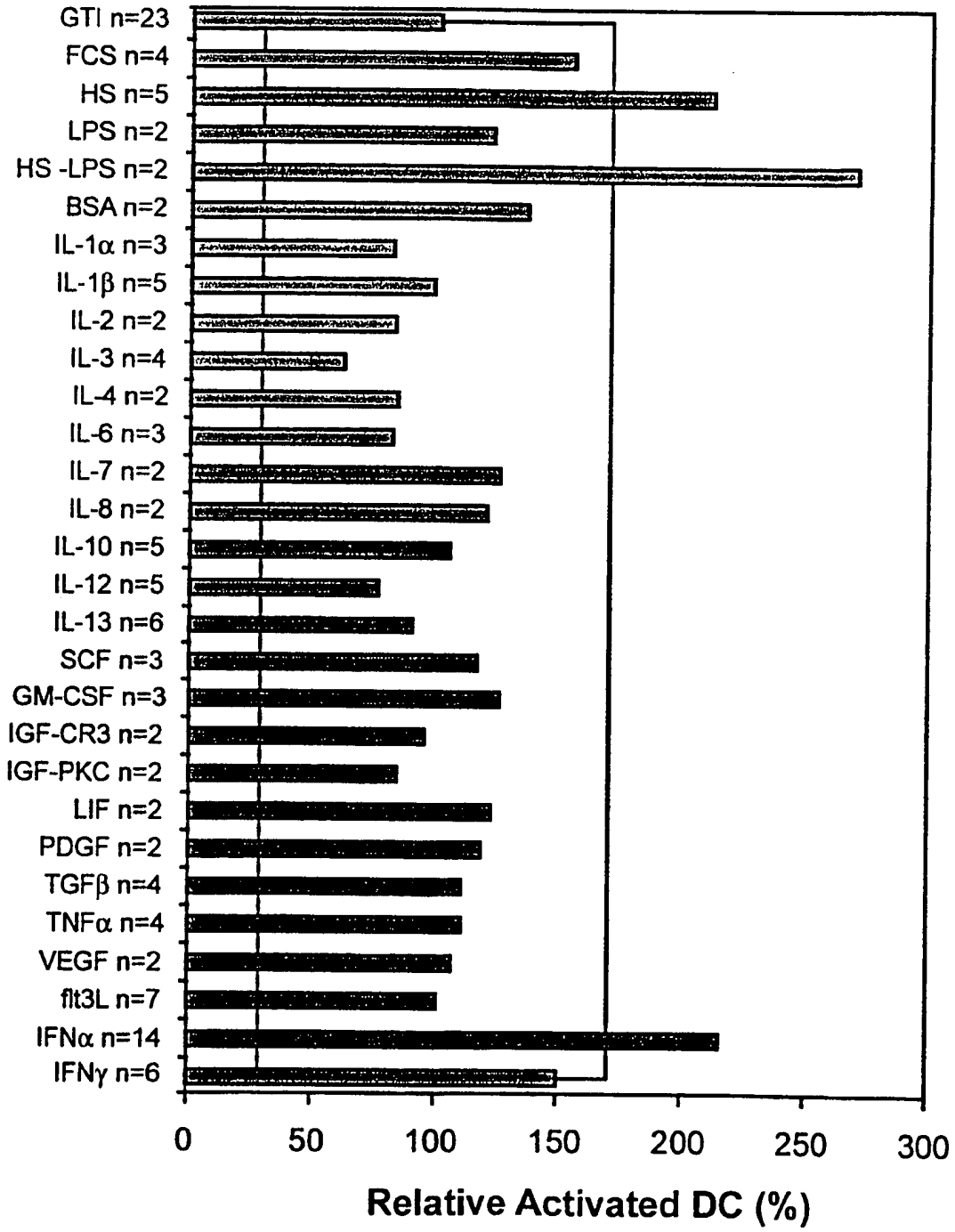

GTI n=23
FCS n=4
HS n=5
LPS n=2
HS -LPS n=2
BSA n=2
IL-1α n=3
IL-1β n=5
IL-2 n=2
IL-3 n=4
IL-4 n=2
IL-6 n=3
IL-7 n=2
IL-8 n=2
IL-10 n=5
IL-12 n=5
IL-13 n=6
SCF n=3
GM-CSF n=3
IGF-CR3 n=2
IGF-PKC n=2
LIF n=2
PDGF n=2
TGFβ n=4
TNFα n=4
VEGF n=2
flt3L n=7
IFNα n=14
IFNγ n=6
0
50
100
150
200
250
300
Relative Activated DC (%)

FIGURE 14

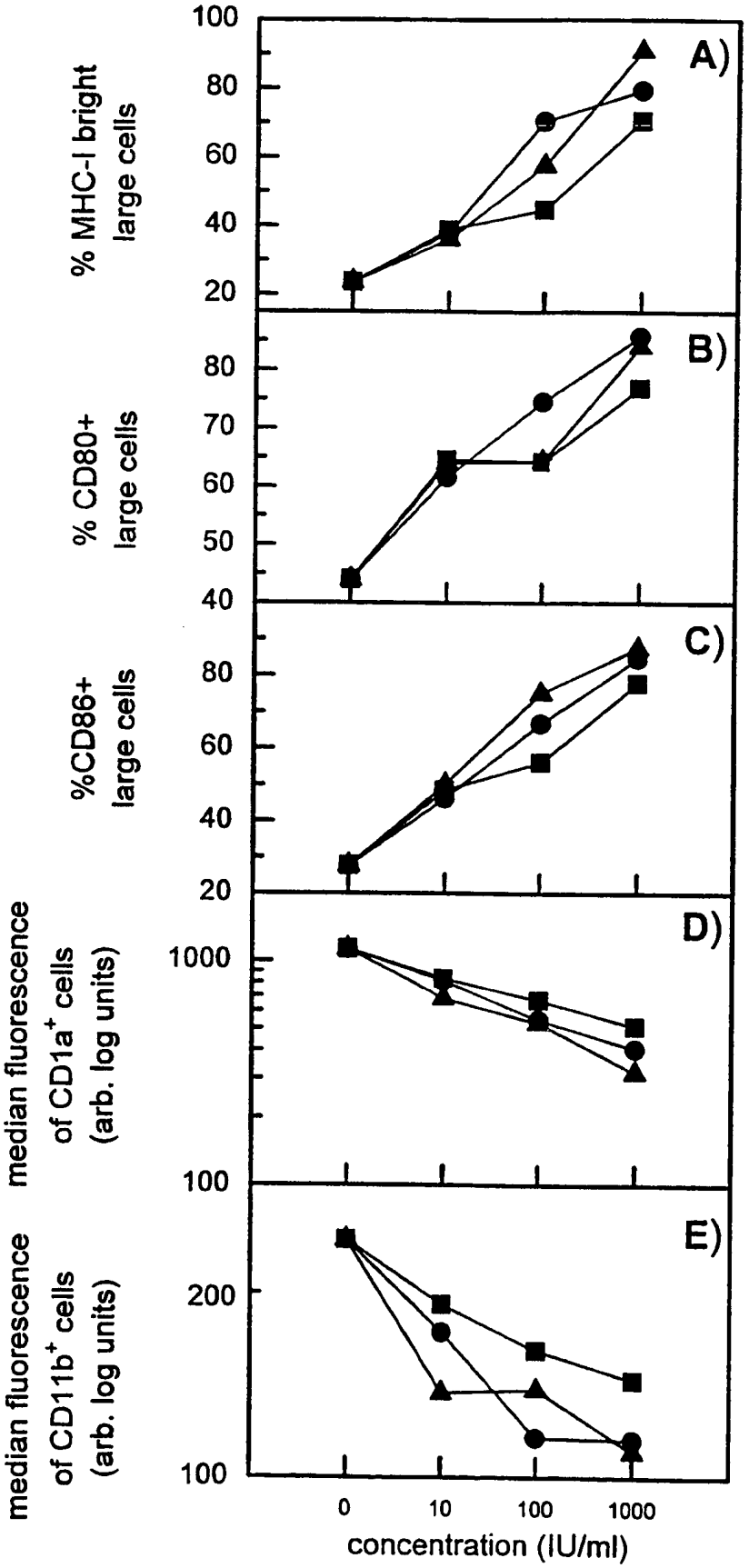

A)
B)
C)
D)
E)
% MHC-I bright large cells
% CD80+ large cells
%CD86+ large cells
median fluorescence of CD1a+ cells (arb. log units)
median fluorescence of CD11b+ cells (arb. log units)
100
80
60
40
20
70
50
1000
200
0
10
100
1000
concentration (IU/ml)

FIGURE 15

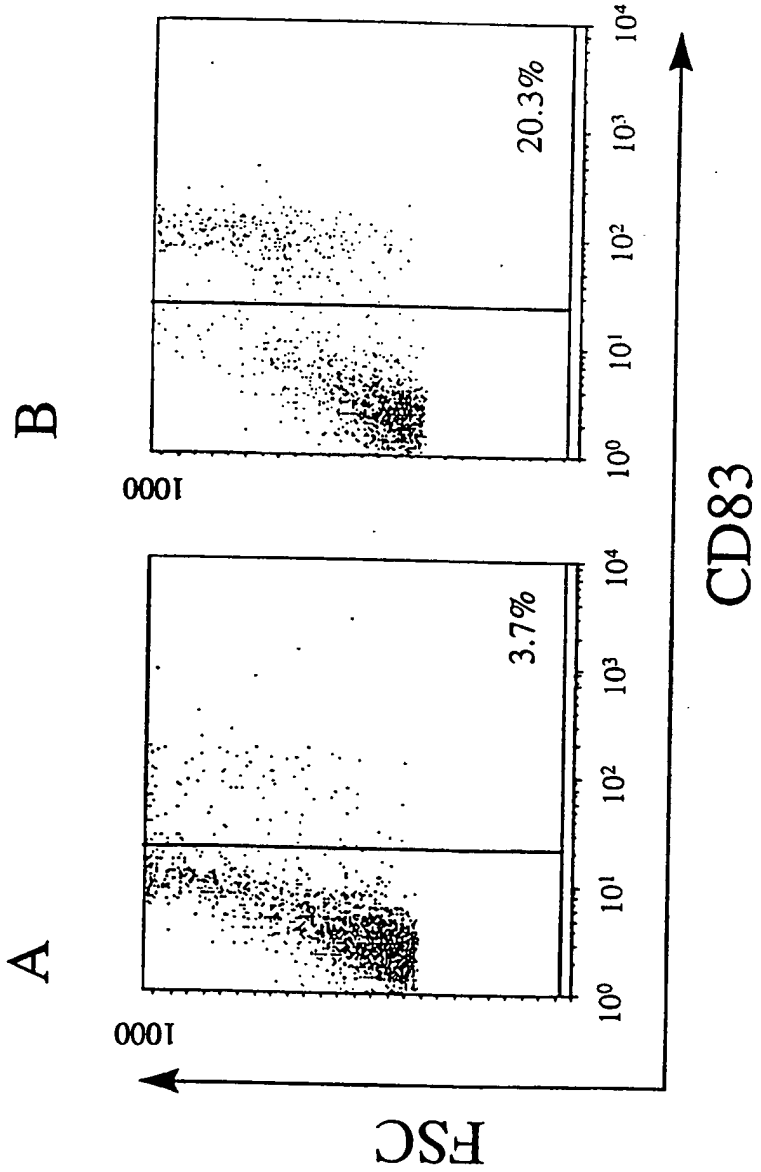


FIGURE 16

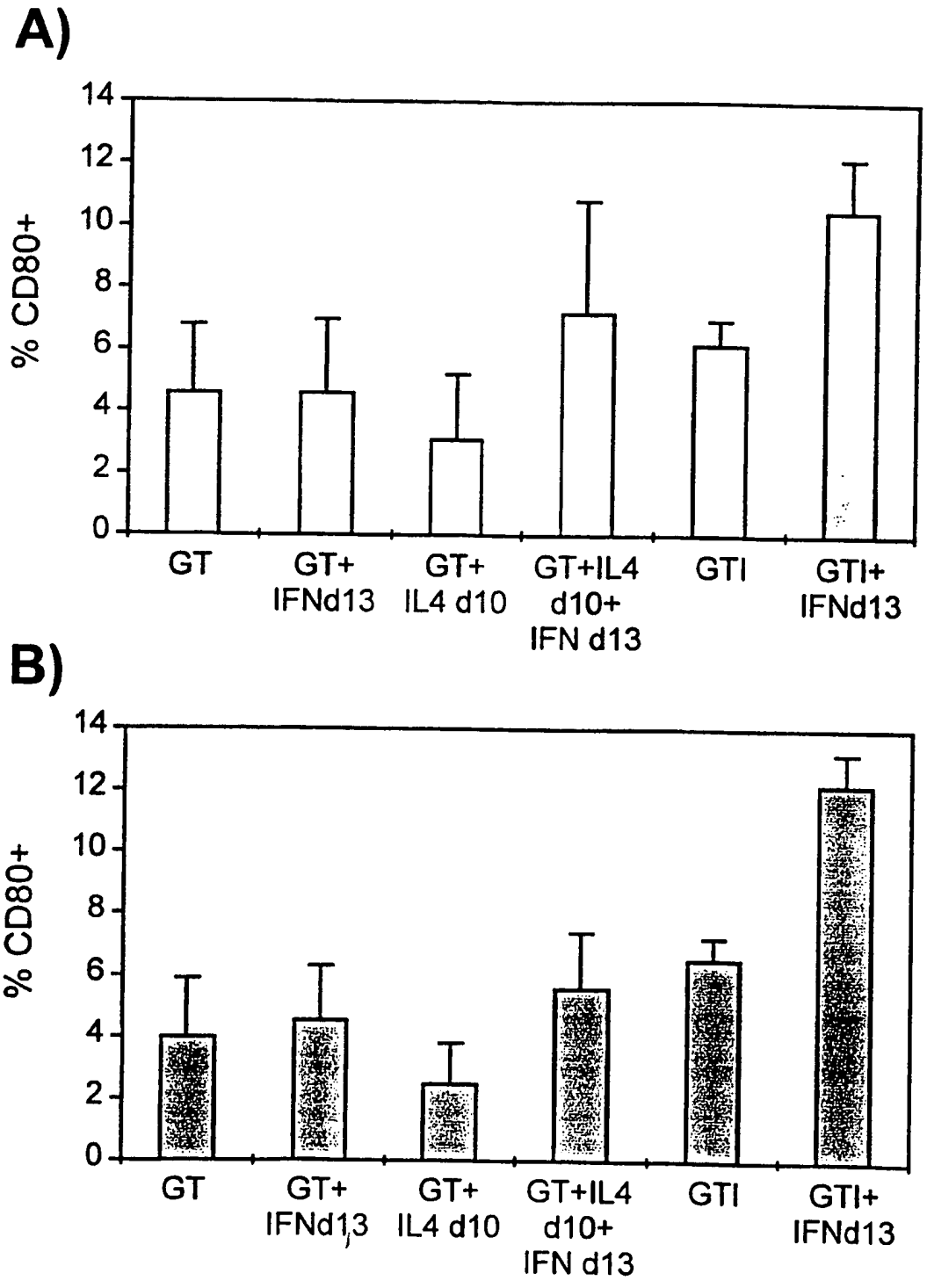
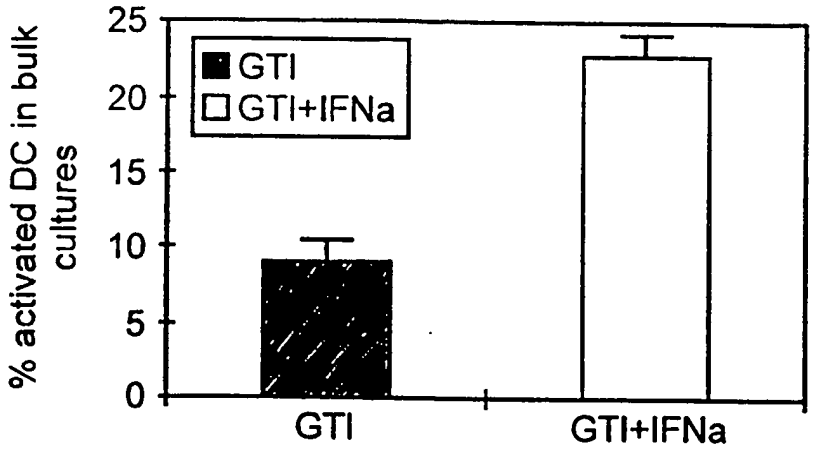

A)
% CD80+
14
12
10
8
6
4
2
0
GT
GT+ IFNd13
GT+ IL4 d10
GT+IL4 d10+ IFN d13
GTI
GTI+ IFNd13
B)
% CD80+
14
12
10
8
6
4
2
0
GT
GT+ IFNd13
GT+ IL4 d10
GT+IL4 d10+ IFN d13
GTI
GTI+ IFNd13

FIGURE 17

Phenotypic response to IFN-a
(n=6 cultures compared in parallel in the MLR)



IFN-a (1000 U/ml) was added to
half of the cultures from day 13 to 16

FIGURE 18

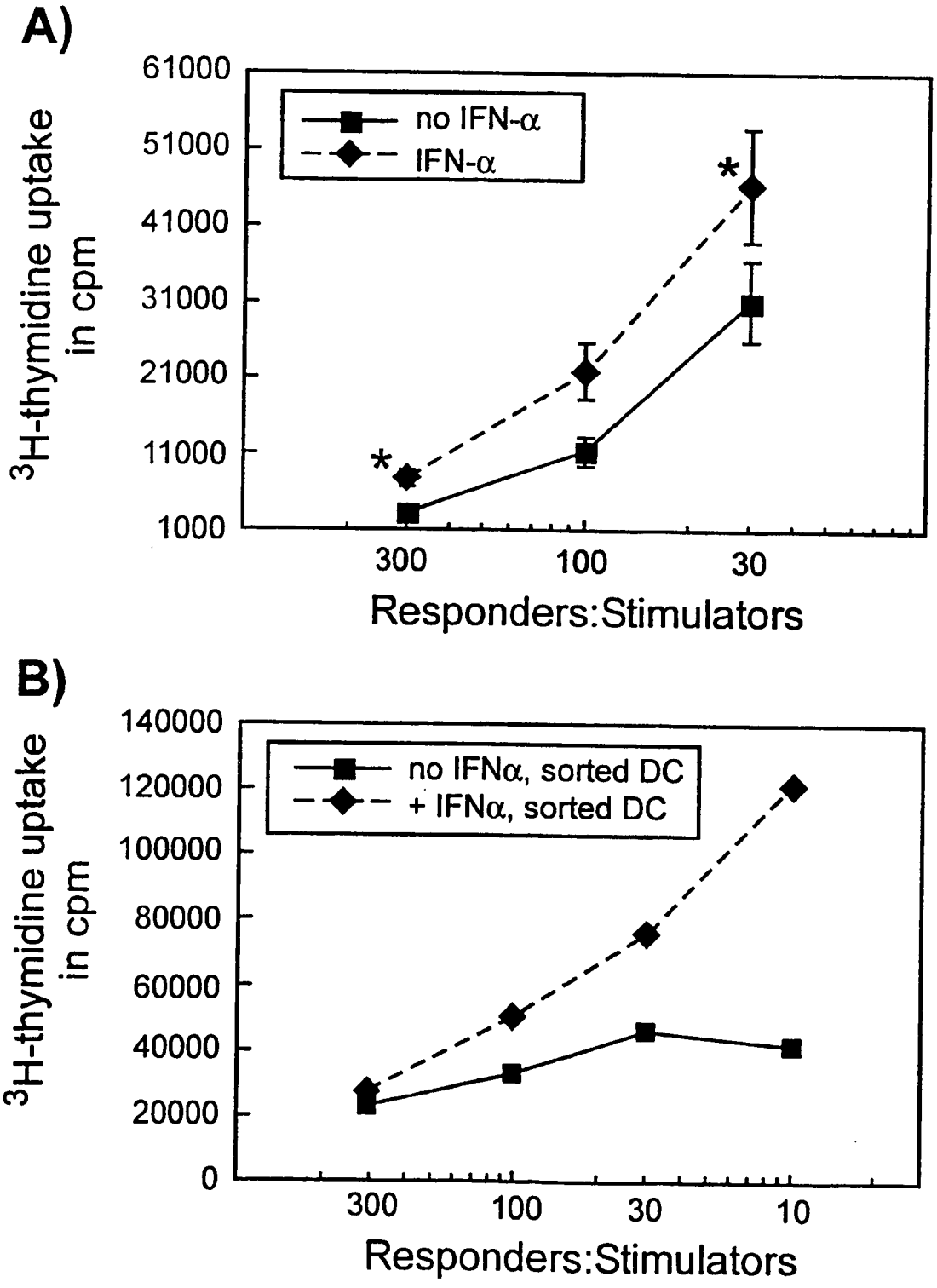
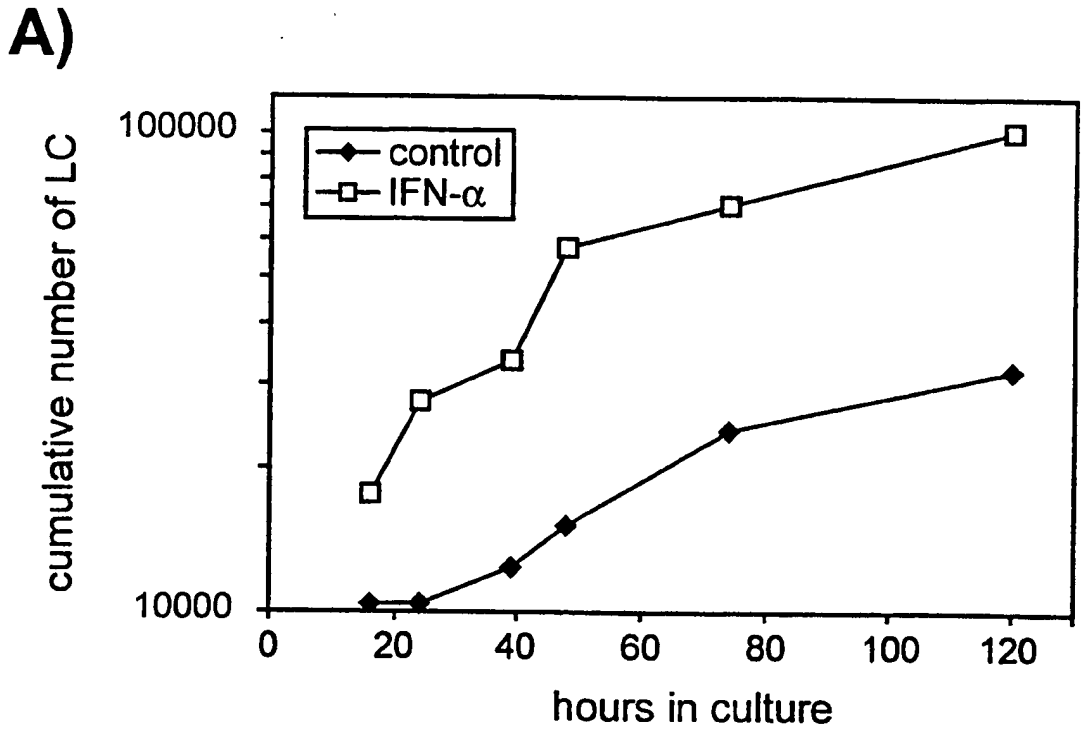

A)
61000
51000
41000
31000
21000
11000
1000
^{3}H-thymidine uptake in cpm
no IFN-α
IFN-α
*
*
300
100
30
Responders:Stimulators
B)
140000
120000
100000
80000
60000
40000
20000
0
^{3}H-thymidine uptake in cpm
no IFNα, sorted DC
+ IFNα, sorted DC
300
100
30
10
Responders:Stimulators

FIGURE 19


A)
100000
10000
cumulative number of LC
control
IFN-α
0
20
40
60
80
100
120
hours in culture

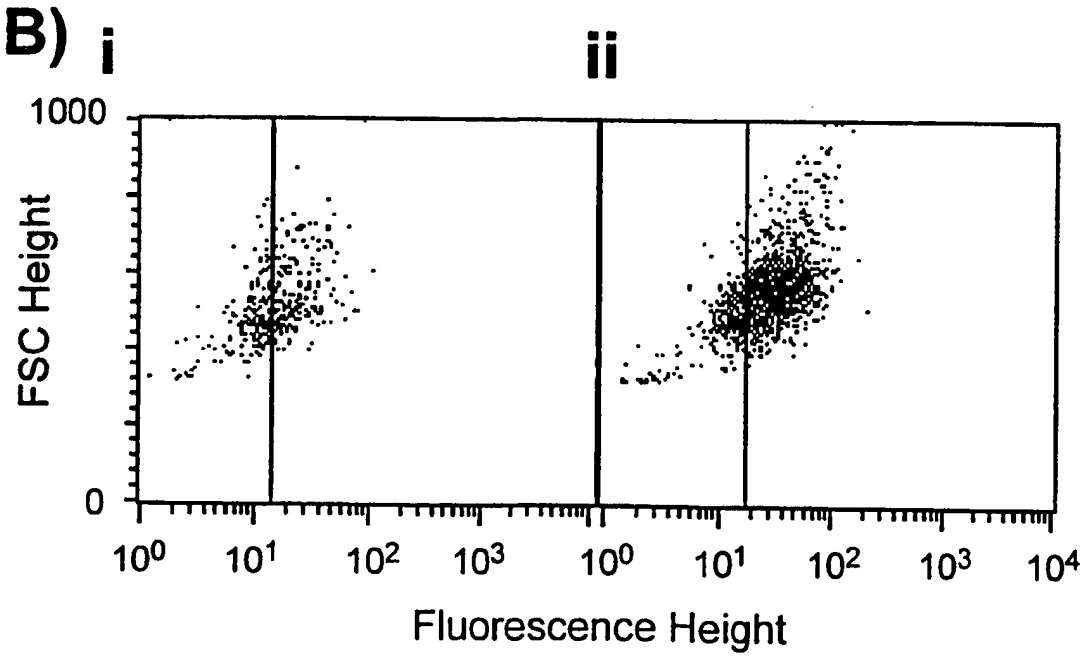

B)
i
ii
1000
0
FSC Height
10^0
10^1
10^2
10^3
10^0
10^1
10^2
10^3
10^4
Fluorescence Height

FIGURE 20

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00801

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 1/38, 5/02, 5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, CHEM ABS, MEDLINE: DENDRITIC CELLS AND GM-CSF AND TNF-ALPHA AND IL-4 AND IFN
(Also searched was the full forms of these abbreviations)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. of INTERFERON RESEARCH, vol. 10(4), 1990, pp 435-446, FELDMAN M and FITZGERALD-BOCARSLY P, "Sequential Enrichment and Immunocytochemical Visualization of Human-α-Producing Cells" Whole article	1-7, 16-39, 52-54
Y	ARCHIVES OF DERMATOLOGICAL RESEARCH, vol. 289, 1996, pp 1-8, JONULEIT H *et al*, "Cytokines and their effects on maturation, differentiation and migration of dendritic cells" Whole article	1-7, 16-39, 52-54
Y	J. EXP. MED., vol. 179, April 1994, pp 1109-1118, SALLUSTO F & LANZAVECCHIA A, "Efficient Presentation of Soluble Antigen by Cultured Human Dendritic Cells Is Maintained by Granulocyte/Macrophage Colony-stimulating Factor Plus Interleukin 4 and Downregulated by Tumor Necrosis Factor α" Whole article	1-7, 16-39, 52-54

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search
23 December 1997

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00801

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	P.N.A.S. USA, vol. 93, March 1996, pp 2588-2592, ZHOU LJ & TEDDER TF, "CD14$^+$ blood monocytes can differentiate into functionally mature CD83$^+$ dendritic cells" Whole article	1-7, 16-39, 52-54
P,Y	AU, A, 73922/96 (IMMUNEX CORPORATION) 10 April 1997 Claims 1-19	1-7, 16-39, 52-54

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU 97/00801

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
AU	73922/96	WO	97/12633

END OF ANNEX